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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C07H 21/00, C12Q 1/68</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 97/12896</b> <b>(43) International Publication Date:</b> 10 April 1997 (10.04.97)
<b>(21) International Application Number:</b> PCT/US96/15934 <b>(22) International Filing Date:</b> 2 October 1996 (02.10.96) <b>(30) Priority Data:</b> 08/539,097 4 October 1995 (04.10.95) US <b>(71) Applicant:</b> EPOCH PHARMACEUTICALS, INC. [US/US]; 1725 220th Street, S.E., No. 104, Bothell, WA 98021 (US). <b>(72) Inventors:</b> KUTYAVIN, Igor, V.; 16520 North Road #B101, Bothell, WA 98012 (US). WOO, Jinsuk; 3730 204th Street S.W. #C-201, Lynnwood, WA 98036 (US). LUKHTANOV, Eugeny, A.; Apartment #E-206, 16520 North Road, Bothell, WA 98012 (US). MEYER, Rich, B., Jr.; 15533 61st Avenue N.E., Bothell, WA 98011 (US). GAMPER, Howard, B.; 14048 212th Drive N.E., Woodinville, WA 98078 (US). <b>(74) Agents:</b> KLEIN, Howard, J. et al.; Klein & Szekeres, L.L.P., Suite 700, 4199 Campus Drive, Irvine, CA 92612 (US).		<b>(81) Designated States:</b> AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> SELECTIVE BINDING COMPLEMENTARY OLIGONUCLEOTIDES  <b>(57) Abstract</b>  In a matched pair of oligonucleotides (ODNs) each member of the pair is complementary or substantially complementary in the Watson Crick sense to a target sequence of duplex nucleic acid where the two strands of the target sequence are themselves complementary to one another. The ODNs include modified bases of such nature that the modified base forms a stable hydrogen bonded base pair with the natural partner base, but does not form a stable hydrogen bonded base pair with its modified partner. This is accomplished when in a hybridized structure the modified base is capable of forming two or more hydrogen bonds with its natural complementary base, but only one hydrogen bond with its modified partner. Due to the lack of stable hydrogen bonding with each other, the matched pair of oligonucleotides have a melting temperature under physiological or substantially physiological conditions of approximately 40 °C or less. However each of the matched ODN pair of the invention forms a substantially stable hybrid with the target sequence in each strand of the duplex nucleic acid. The hybrids of target duplex nucleic acids formed with the ODN pairs of the invention are useful for gene mapping and in diagnostic and therapeutic applications.		

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1                   SELECTIVE BINDING COMPLEMENTARY  
2   OLIGONUCLEOTIDES

3                   BACKGROUND OF THE INVENTION  
4

5           1.   Field of the Invention

6           The present invention is directed to oligonucleotides which  
7   include modified bases such that members of a matched pair of the  
8   oligonucleotides are unable to form stable hybrids with one another and  
9   yet are able to form stable, sequence specific hybrids with  
10   complementary unmodified DNA or RNA strands. The present  
11   invention is also directed to the use of such oligonucleotides as an  
12   anti-sense and anti-gene agents and probes for specific sequences in  
13   single or double stranded DNA or RNA.

14          2.   Brief Description of Prior Art

15          It is well known that oligonucleotides (ODNs) do not readily  
16   hybridize to complementary sequences in double stranded DNA or in  
17   DNA or RNA secondary structure. Nevertheless, it is also known that  
18   the ability to sequence specifically access double stranded DNA or  
19   single stranded RNA or DNA in secondary structure would have great  
20   utility in gene mapping, diagnostics and therapeutic applications.  
21   Methods known in the prior art which, although limited in scope,  
22   accomplish hybridization of ODNs to duplex nucleic acids include  
23   triplex formation (see Troel, S. et al. Science 1991, 254, 1639), the  
24   branch capture reaction (Weinstock, P. et al. Nucl. Acids Res. 1990, 18,  
25   4207), recombinase mediated synapsis (Roca, A. I.; et al. Rev. Biochem.  
26   Mol. Biol. 1990, 25, 415) and cross-linking of the hybridized ODN to at  
27   least one strand of the duplex nucleic acid (PCT application WO  
28   93/03736, published March 4, 1993).

29          There is however still a significant need, and room for  
30   improvement in the art, for oligonucleotides which are able to sequence  
31   specifically hybridize to duplex nucleic acids. The present invention  
32   provides such oligonucleotides.

**SUMMARY OF THE INVENTION**

In accordance with the present invention a matched pair of oligonucleotides (ODNs) are provided where each member of the pair is complementary or substantially complementary in the Watson Crick sense to a target duplex sequence. However the ODNs include modified bases of such nature that the modified base forms stable hydrogen bonded base pairs with the natural partner base, but does not form stable hydrogen bonded base pairs with its modified partner. Generally speaking, this is accomplished when in a hybridized structure the modified base is capable of forming two or more hydrogen bonds with its natural complementary base, but only one or no hydrogen bonds with its modified partner. Thus, the matched pair of oligonucleotides in accordance with the present invention do not form substantially stable hydrogen bonded hybrids with one another, as manifested in a melting temperature (under physiological or substantially physiological conditions) of approximately 40°C or less. The ODNs of the invention, however, form substantially stable hybrids with the target sequence in each strand of duplex nucleic acid. Due to the increased (approximately double) number of hydrogen bonds in such hybrids (when compared to hybrids that would be formed between a single ODN and duplex nucleic acid) the hybrids formed with the ODN pairs of the present invention are more stable, and lend themselves for gene mapping, diagnostic and therapeutic use. The ODNs of the present invention are termed Selective Binding Complementary (SBC) ODNs, and may be referred to under that name in this application for patent.

The SBC ODNs of the present invention may optionally be connected to one another with a covalent "tether" of such nature that the tether does not prevent hybridization of each ODN to one strand of the target sequence. The SBC ODNs of the present invention may optionally include modifications in the sugar moiety, in the phosphate backbone, and may have cross-linking groups and/or reporter groups

1 attached.

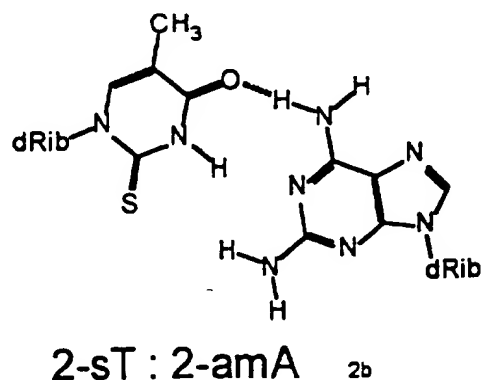
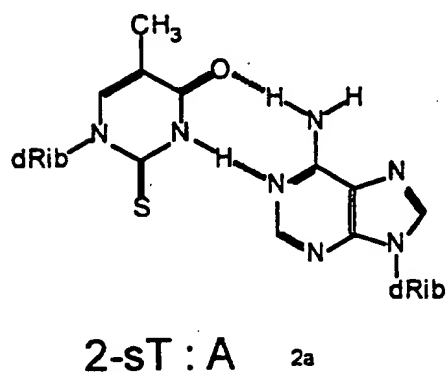
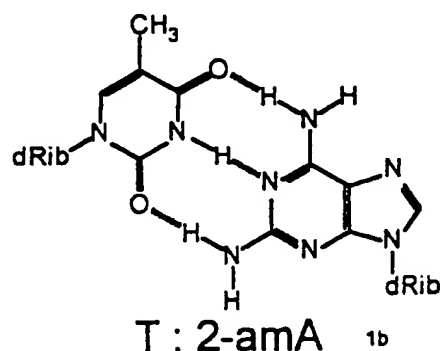
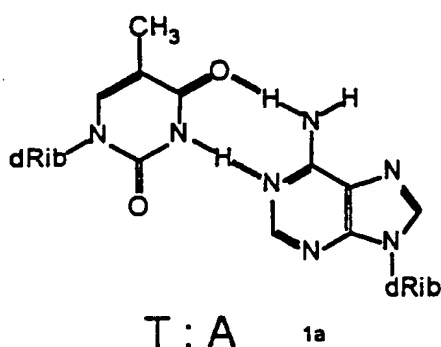
## 2 DETAILED DESCRIPTION OF THE INVENTION

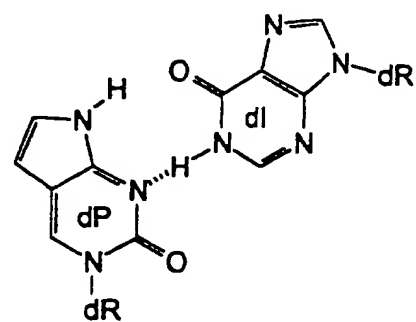
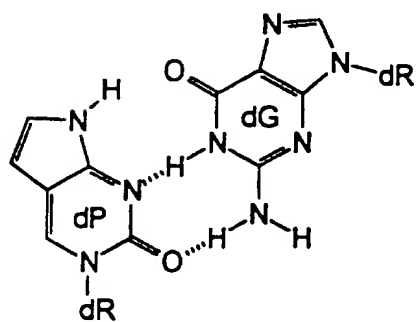
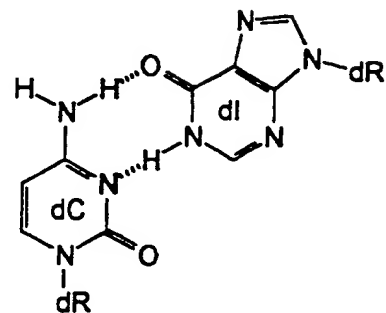
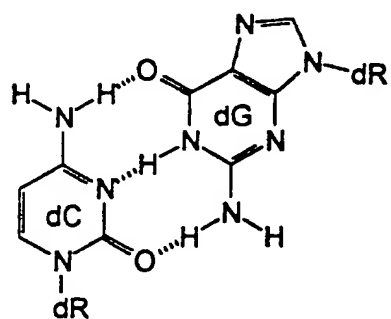
3 As is noted in the Summary of the present application, a key  
4 feature of the SBC ODNs of the present invention is that each one of a  
5 matched pair of the SBC ODNs is complementary, or substantially  
6 complementary, to one target sequence in duplex nucleic acid wherein  
7 the target sequences are themselves complementary or substantially  
8 complementary to one another, and each one of the matched pair of  
9 SBC ODNs forms a stable hydrogen bonded hybrid with one strand of  
10 the target sequence. Due to the presence of modified bases in the SBC  
11 ODN, although these ODNs are complementary to one another, they  
12 are unable to form a stable hydrogen bonded hybrid, as manifested by a  
13 melting temperature of approximately 40°C or less. Thus, the SBC  
14 ODNs are not hybridized to one another but they readily hybridize,  
15 especially in the presence of recombinase enzymes when the target is in  
16 long double stranded DNA, with both strands of the target sequence.

17 In accordance with well established convention in the art, the  
18 naturally occurring nucleotide components of nucleic acids have the  
19 designation A, U, G and C, (RNA) and dA, dT, dG and dC (DNA).  
20 As it will become apparent from the following description, the present  
21 invention applies to both ribonucleotides and deoxyribonucleotides, and  
22 therefore, unless the context otherwise requires, no distinction needs to  
23 be made in this description between A and dA, U and dT, etc.

24 Analogs of A which are modified in the base portion to form in  
25 an ODN-to-nucleic acid or ODN-to-ODN interaction a stable hydrogen  
26 bonded pair with T, (or U in the case of RNA) but not with T' are  
27 designated A'. Analogs of T which are modified in the base portion to  
28 form in an ODN-to-nucleic acid or ODN-to-ODN interaction a stable  
29 hydrogen bonded pair with A, but not with A' are designated T'.  
30 Analogs of G which are modified in the base portion to form in an

1 ODN-to-nucleic acid or ODN-to-ODN interaction a stable hydrogen  
2 bonded pair with C, but not with C' are designated G'. Analogs of C  
3 which are modified in the base portion to form in an ODN-to-nucleic  
4 acid or ODN-to-ODN interaction a stable hydrogen bonded pair with  
5 G, but not with G' are designated C'. The foregoing conditions are  
6 satisfied when each of the A', T', G' and C' nucleotides (collectively the  
7 modified SBC nucleotides) form, in an ODN-to-nucleic acid or  
8 ODN-to-ODN interaction, two or more hydrogen bonds with their  
9 natural partner, but only one or no hydrogen bonds with their modified  
10 SBC nucleotide partner. This is illustrated by Formulas 1a, 1b, 2a, 2b,  
11 3a, 3b, 4a and 4b where the hydrogen bonding between natural A-T (or  
12 A-U in case of RNA) and G-C pairs, and hydrogen bonding between  
13 exemplary A'-T, T'-A, G'-C, C'-G, A'-T' and G'-C' pairs are illustrated.







1           A sufficient number of the modified SBC nucleotides are  
2   incorporated such that complementary positions in both SBC ODNs are  
3   modified into a matched pair of SBC ODNs of the present invention so  
4   that the pair of the matched set does not form a stable hybrid; in other  
5   words under physiological conditions it has a melting temperature of  
6   approximately 40°C or less. It is not necessary to replace each natural  
7   nucleotide of the ODN with a modified SBC nucleotide in order to  
8   accomplish this. Both members of the matched pair are however  
9   complementary to a target sequence in double stranded or duplex  
10   nucleic acid, where the two strands or parts of the target duplex are  
11   themselves complementary or substantially complementary to one  
12   another. As it is described in more detail below, an important use of  
13   the SBC ODNs of the present invention is hybridization with secondary  
14   structure of mRNA wherein the mRNA itself forms a duplex, such as in  
15   hairpin loops. It is known that secondary structure of mRNA and  
16   ribosomal RNA do not have two strands in the strict sense of that term.  
17   Nevertheless, unless the context otherwise indicates, in the present  
18   description the terminology "two strands" of double stranded nucleic  
19   acids also refers to the two complementary portions of duplex mRNA  
20   or of duplex ribosomal RNA as well. The general concept of double  
21   stranded DNA and of secondary structure in mRNA and ribosomal  
22   RNA is covered in this description by the term "duplex nucleic acid".  
23   The term "RNA" can apply to any functional RNA in living organisms,  
24   such as messenger, transfer, ribosomal, small nuclear, guide, genomic,  
25   etc. RNA.

26           Generally speaking, the SBC ODNs of the present invention  
27   include, in addition to the modified SBC nucleotides, the naturally  
28   occurring nucleotides, and may also include some other minor naturally  
29   occurring or chemically modified nucleotides, as long as such  
30   modifications do not interfere significantly with the complementary

1 binding ability of the ODN, as discussed above. Certain important  
2 embodiments of the SBC ODNs of the present invention include  
3 reporter groups and or cross linking functions covalently attached to one  
4 or more nucleotides of the ODN. These embodiments are described in  
5 detail below. The SBC ODNs of the present invention may include  
6 pentofuranose moieties other than ribose or 2-deoxyribose, as well as  
7 derivatives of ribose and 2-deoxyribose, for example  
8 3-amino-2-deoxyribose, 2-fluoro-2-deoxyribose, and 2-Q-C<sub>1-6</sub> alkyl or  
9 2-Q-allyl ribose, particularly 2-Q-methyl ribose. The glycosidic linkage  
10 may be of the  $\alpha$  or  $\beta$  configuration, with the  $\beta$  configuration being  
11 preferred. The phosphate backbone of the SBC ODNs of the present  
12 invention may include phosphorothioate linkages. Moreover,  
13 cross-linking agents, reporter groups, lipophilic groups (including  
14 cholesterol and related "steroid" derivatives) intercalators, minor groove  
15 binders as well as alkyl, hydroxy-alkyl, or amino-alkyl tails can also be  
16 attached to the 3'- or 5'- phosphate end of the SBC ODNs.

17 The number of nucleotide building units in the SBC ODNs of the  
18 present invention is not critical and is generally speaking, in the range  
19 of approximately 5 to 99.

20 A general structure for a preferred class of the modified A  
21 analog, A', within the scope of the invention and shown as a  
22 3'-phosphate (or phosphorothioate) incorporated into the SBC ODN, is  
23 provided by Formulas 5, 6 and 7, wherein

24 X is N or CH;

25 Y is O or S;

26 Z is OH or CH<sub>3</sub>;

27 R is H, F, or OR<sub>2</sub>, where R<sub>2</sub> is C<sub>1-6</sub> alkyl or allyl, or H in case of  
28 RNA, and

29 R<sub>1</sub> is C<sub>1-4</sub> alkyl, C<sub>1-4</sub> alkoxy, C<sub>1-4</sub> alkylthio, F, or NHR<sub>3</sub>, where R<sub>3</sub>  
30 is H, or C<sub>1-4</sub> alkyl, and where the 8 position of the purine, the 3

1 position of the pyrazolopyrimidine or the 5 position of the pyrro-  
 2 lopyrimidine optionally serve as point of attachment for a cross-linkig  
 3 function, or reporter group as described below. A preferred  
 4 embodiment of the SBC nucleotide A' has 2,6-diaminopurine  
 5 (2-aminoadenine) as the base, as shown in Formula 1b. The latter  
 6 nucleotide is abbreviated as 2-amA or d2-amA as applicable.

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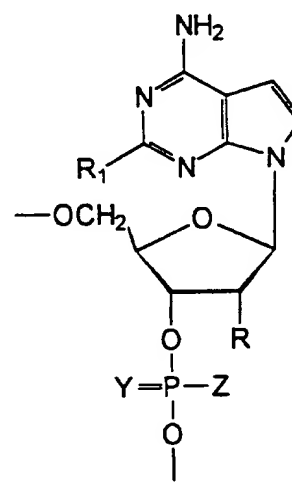
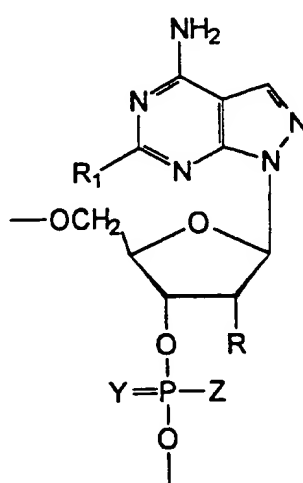
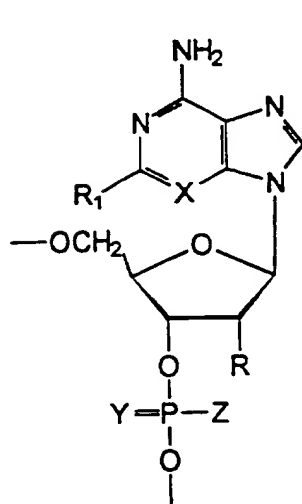
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**Formula 5****Formula 6****Formula 7**

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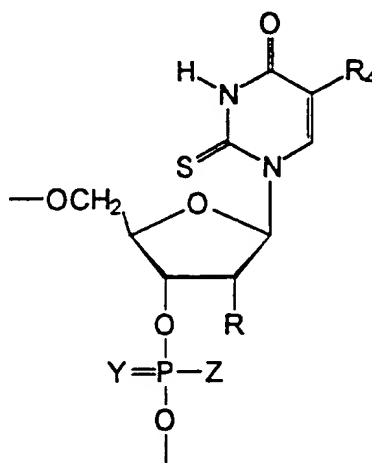
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A general structure for a preferred class of the modified T  
 analog, T', within the scope of the invention and shown as a  
 3'-phosphate (or phosphorothioate) incorporated into the SBC ODN, is  
 provided by Formula 8, wherein

Y, Z and R are defined as above, and

R<sub>4</sub> is H, C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkenyl, C<sub>1-6</sub> alkynyl, or optionally the  
 5-position of the pyrimidine serves as point of attachment for a  
 cross-linking function, or a reporter group as described below. A  
 preferred embodiment of the SBC nucleotide T' has

1 2-thio-4-oxo-5-methylpyrimidine (2-thiothymine) as the base, as shown  
 2 in Formula 2b. The latter nucleotide is abbreviated as 2-sT or d 2-sT as  
 3 applicable.



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18 **Formula 8**

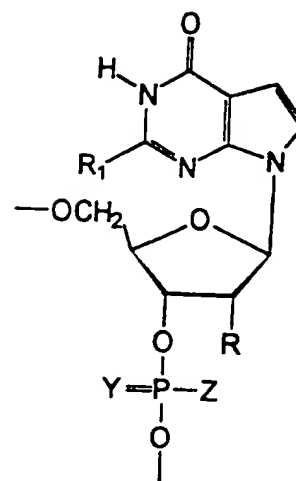
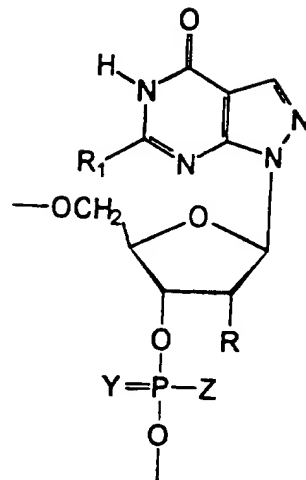
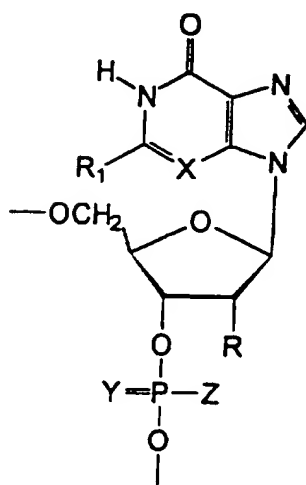
19 A general structure for a preferred class of the modified G  
 20 analog, G', within the scope of the invention and shown as a  
 21 3'-phosphate (or phosphorothioate) incorporated into the SBC ODN, is  
 22 provided by Formulas 9, 10 and 11, wherein

23  $R_1$  is H,  $C_{1-4}$  alkyl,  $C_{1-4}$  alkoxy,  $C_{1-4}$  alkylthio, F or  $NHR_2$ , where  $R_2$   
 24 is defined as above,

25 X, Y, Z and R are defined as above, and the 8 position of the  
 26 purine, the 3 position of the pyrazolopyrimidine or the 5 position of  
 27 the pyrrolopyrimidine optionally serve as point of attachment for a  
 28 cross-linking agent, or reporter group as described below. A preferred  
 29 embodiment of the SBC nucleotide G' has 6-oxo-purine (hypoxanthine)  
 30 as the base, as shown in Formula 3b. The latter nucleotide is

1 abbreviated as I or dI as applicable.

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17 **Formula 9**

**Formula 10**

**Formula 11**

18 A general structure for a preferred class of the modified C  
19 analog, C', within the scope of the invention and shown as a  
20 3'-phosphate (or phosphorothioate) incorporated into the SBC ODN, is  
21 provided by **Formulas 12 and 13**, wherein

22 Y, Z, R and R<sub>4</sub> are defined as above, or optionally the 5-position  
23 of the pyrimidine serves as point of attachment for a cross-linking  
24 function, or a reporter group as described below;

25 Z<sub>1</sub> is O or NH, and

26 R<sub>3</sub> is H or C<sub>1-4</sub> alkyl.

27 A preferred embodiment of the SBC nucleotide C' has  
28 pyrrolo-[2,3-d]pyrimidine-2(3H)-one as the base, as shown in **Formula**  
29 **4b**. The latter nucleotide is abbreviated as P or dP as applicable.

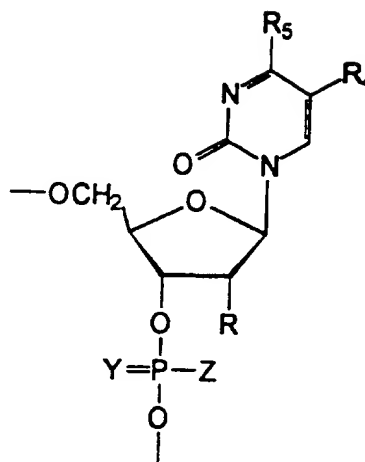
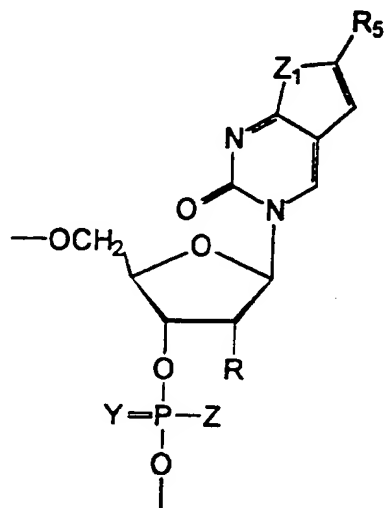
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1 **SBC ODNs bearing a cross-linking group**

2       An important class of the SBC ODNs of the present invention  
3 bear a cross-linking function or group. The cross-linking function or  
4 group may be attached to a nucleotide which is itself an SBC nucleotide  
5 (as defined above) or to another type of "natural" or modified  
6 nucleotide, and the attachment may be to the heterocyclic base, to the  
7 sugar or to a phosphate, preferably a terminal phosphate moiety. The  
8 cross linking group or function serves the purpose that after  
9 hybridization of the SBC ODN to a target sequence of duplex nucleic  
10 acid, the cross-linking function covalently links the SBC ODN to the  
11 target. As it will be readily recognized by those skilled in the art,  
12 covalent cross-linking increases the efficiency and effectiveness of the  
13 SBC ODNs as probes for diagnostic, analytical or other investigative  
14 purposes, and also as therapeutic anti-sense and anti-gene agents. A  
15 cross-linking group or function may be attached to one or both  
16 members of a matched pair of SBC ODNs, and consequently one or  
17 both strands of the target sequence may become covalently bonded  
18 (alkylated) by this class of SBC ODNs.

19       In light of the foregoing, the cross-linking agents incorporated in  
20 the present invention meet the requirements that (1) each cross-linking  
21 agent is covalently bonded to a site on the SBC ODN, (2) its length and  
22 steric orientation is such that it reaches a suitable reaction site in the  
23 target sequence after the SBC ODN is hybridized or complexed with the  
24 target and (3) has a reactive group which reacts with a reactive  
25 nucleophilic group of the target sequence.

26       In the simplest terms the cross-linking agent itself may  
27 conceptually be divided into two groups or moieties, namely the reactive  
28 group, which is typically and preferably an electrophilic leaving group  
29 (L), and an "arm" (A\*) which attaches the leaving group L to the  
30 respective site on the SBC ODN. The leaving group L may be chosen



Formula 12

Formula 13

As noted above, the SBC ODNs of the present invention are utilized in a matched pair where the members of the pair are not covalently linked to one another. In an alternative embodiment the two members of the matched pair may be covalently linked (tethered) to one another with a covalent linkage that does not participate in hybridization and does not prevent hybridization of the two members (each of which is complementary to one target sequence of duplex nucleic acid) to the two strands of the target sequence. Linking groups which are suitable as "tethers" for linking the two SBC ODNs of a matched pair to one another include approximately selected (to avoid hydrogen bonding) nucleotide sequences having approximately 1 to 10 nucleotides. A specific example for the tether would be an ODN moiety having four T's. Alternatively the tethering linkage may comprise the grouping  $-\text{[OCH}_2\text{-CH}_2\text{]}_n\text{-O-}$ , where  $n$  is 1 to 10.

1 from, for example, such groups as chloro, bromo, iodo,  $\text{SO}_2\text{R}''$ , or  
2  $\text{S}^+\text{R}''\text{R}'''$ , where each of  $\text{R}''$  and  $\text{R}'''$  is independently  $\text{C}_{1-6}$ alkyl or aryl or  
3  $\text{R}''$  and  $\text{R}'''$  together form a  $\text{C}_{1-6}$ alkylene bridge. Chloro, bromo and  
4 iodo are preferred. Within these groups haloacetyl groups such as  
5  $-\text{COCH}_2\text{I}$ , and bifunctional "nitrogen mustards", such as  $-\text{N}-[(\text{CH}_2)_2-\text{Cl}]_2$   
6 are preferred. The leaving group will be altered by its leaving ability.  
7 Depending on the nature and reactivity of the particular leaving group,  
8 the group to be used is chosen in each case to give the desired  
9 specificity to the irreversibly binding probes or chemotherapeutic agents.

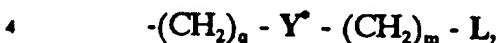
10 Although as noted above the "arm" (or linker arm)  $\text{A}^*$  may  
11 conceptually be regarded as a single entity which covalently bonds the  
12 SBC ODN to the leaving group L, and maintains the leaving group L at  
13 a desired distance and steric position relative to the SBC ODN, in  
14 practice the "arm"  $\text{A}^*$  may be constructed in a synthetic scheme where a  
15 bifunctional molecule is covalently linked to the SBC ODN (for example  
16 by a phosphate ester bond to the 3' or 5' terminus, or by a  
17 carbon-to-carbon bond to a heterocyclic base) through its first  
18 functionality, and is also covalently linked through its second  
19 functionality (for example an amine) to a "hydrocarbyl bridge" (alkyl  
20 bridge, alkylaryl bridge or aryl bridge, or the like) which, in turn, carries  
21 the leaving group.

22 A general formula of the cross linking function is thus  $-\text{A}^*-\text{L}$ , or  
23  $-\text{A}^*-\text{L}_2$ , where L is the above defined leaving group and  $\text{A}^*$  is a moiety  
24 that is covalently linked to the SBC ODN. The  $\text{A}^*$  "arm" moiety itself  
25 should be unreactive (other than through the leaving group L) under  
26 the conditions of hybridization of the SBC ODN with the target nucleic  
27 acid sequence, and should maintain the leaving group L in a desired  
28 steric position and distance from the desired site of reaction such as an  
29 N-7 position of a guanosine residue in the target sequence. Generally  
30 speaking, the length of the  $\text{A}^*$  group should be equivalent to the length



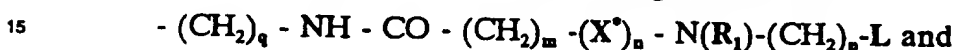
1 of a normal alkyl chain of approximately 2 to 50 carbons.

2 An exemplary more specific formula for a class of preferred  
3 embodiments of the cross-linking function is



5 where L is the leaving group, defined above, each of m and q is  
6 independently 0 to 8, inclusive, and where Y\* is defined as a "functional  
7 linking group". A "functional linking group" is a group that has two  
8 functionalities, for example -NH<sub>2</sub> and -OH, or -COOH and -OH, or  
9 -COOH and -NH<sub>2</sub>, which are capable of linking the (CH<sub>2</sub>)<sub>q</sub> and (CH<sub>2</sub>)<sub>m</sub>  
10 bridges. An acetylenic terminus (HC≡C-) is also a suitable functionality  
11 as a precursor for Y\*, because it can be coupled to certain heterocycles  
12 and thereafter hydrogenated, as described below.

13 Other exemplary and more specific formulas for a class of  
14 preferred embodiments of the cross-linking function are



17 where q, m and L are defined as above, q' is 3 to 7 inclusive, q" is  
18 1 to 7 inclusive, X\* is phenyl or simple substituted phenyl (such as  
19 chloro, bromo, lower alkyl or lower alkoxy substituted phenyl), n is 0 or  
20 1, p is an integer from 1 to 6, and R<sub>1</sub> is H, lower alkyl or (CH<sub>2</sub>)<sub>p</sub>-L.  
21 Preferably p is 2. Those skilled in the art will recognize that the  
22 structure - N(R<sub>1</sub>)-(CH<sub>2</sub>)<sub>2</sub>-L describes a "nitrogen mustard", which is a  
23 class of potent alkylating agents. Particularly preferred within this class  
24 of SBC ODNs of the invention are those where the cross-linking agent  
25 includes the functionality - N(R<sub>1</sub>)-(CH<sub>2</sub>)<sub>2</sub>-L where L is halogen,  
26 preferably chlorine; and even more preferred within this class are those  
27 modified SBC ODNs where the cross linking agent includes the  
28 grouping - N-[(CH<sub>2</sub>)<sub>2</sub>-L]<sub>2</sub> ( a "bifunctional" N-mustard).

29 A particularly preferred partial structure of the cross linking  
30 agent includes the grouping



2   In a particularly preferred embodiment the just-noted cross-linking  
3   group is attached to an n-hexylamine bearing tail at the 5' and 3' ends  
4   of the SBC ODN in accordance with the following structure:



6   where R' signifies the terminal 5' or 3'-phosphate group of the SBC  
7   ODN.

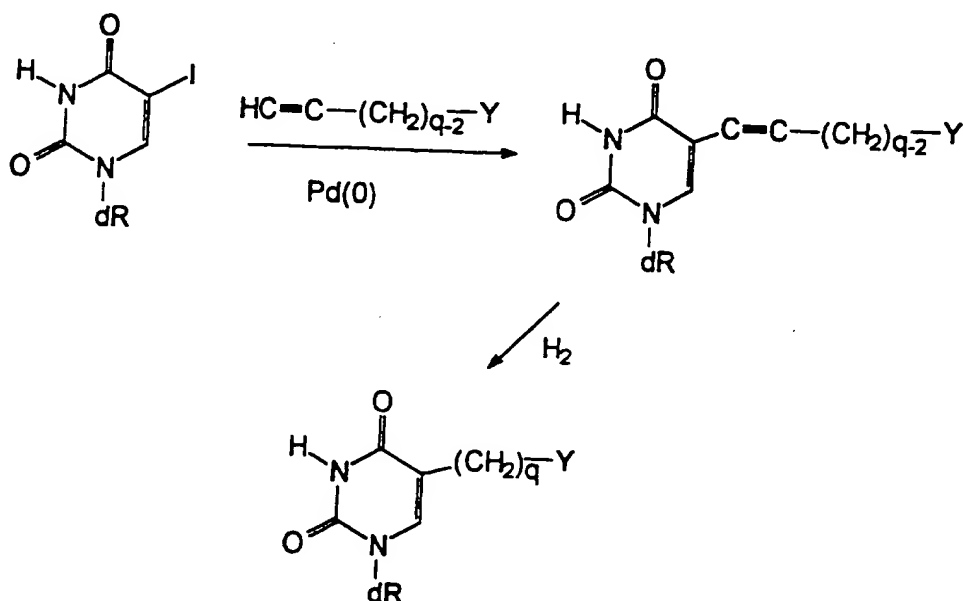
8           Other examples for the A\*-L group, particularly when attached to  
9   a heterocyclic base in the oligonucleotide (such as to the 5-position of  
10   2'-deoxyuridine) are 3-iodoacetamidopropyl,  
11   3-(4-bromobutyramido)propyl, 4-iodoacetamidobutyl and  
12   4-(4-bromobutyramido)butyl groups.

13           In accordance with other preferred embodiments, the  
14   cross-linking functionality is covalently linked to the heterocyclic base,  
15   for example to the uracil moiety of a 2'-deoxyuridylic acid building  
16   block of the SBC ODN. The linkage can occur through the  
17   intermediacy of an amino group, that is, the "arm-leaving group  
18   combination" (A\*-L) may be attached to a 5-amino-2'-deoxyuridylic acid  
19   building unit of the SBC ODN. In still other preferred embodiments  
20   the "arm-leaving group combination" (A\*-L) is attached to the 5-position  
21   of the 2'-deoxyuridylic acid building unit of the SBC ODN by a  
22   carbon-to-carbon bond. Generally speaking,  
23   5-substituted-2'-deoxyuridines can be obtained by an adaptation of the  
24   general procedure of Robins et al. (Can. J. Chem., 60:554 (1982); J.  
25   Org. Chem., 48:1854 (1983)), as shown in Reaction Scheme 1. In  
26   accordance with this adaptation, the palladium-mediated coupling of a  
27   substituted 1-alkyne to 5-iodo-2'-deoxyuridine gives an  
28   acetylene-coupled product. The acetylenic dUrd analog is reduced, with  
29   Raney nickel for example, to give the saturated compound, which is  
30   then used for direct conversion to a reagent for use on an automated

1 DNA synthesizer, as described below. In Reaction Scheme 1, q is  
2 defined as above, and Y' is either Y\* (as defined above) or is a suitable  
3 protected derivative of Y\*. Y' can also be defined as a group which  
4 terminates in a suitably protected nucleophilic function, such as a  
5 protected amine. Examples of reagents which can be coupled to  
6 5-iodo-2'-deoxyuridine in accordance with this scheme are  
7  $\text{HC}\equiv\text{CCH}_2\text{OCH}_2\text{CH}_2\text{N}(\text{CO})_2\text{C}_6\text{H}_4$  (phtalimidoethoxypropyne),  
8  $\text{HC}\equiv\text{CCH}_2\text{OCH}_2\text{CH}_2\text{NHCOCF}_3$  (trifluoroacetamidoethoxypropyne),  
9  $\text{HC}\equiv\text{CCH}_2\text{N}(\text{CO})_2\text{C}_6\text{H}_4$  (phtalimidopropyne) and  $\text{HC}\equiv\text{CCH}_2\text{NHCOCF}_3$ ,  
10 (trifluoroacetamidopropyne),

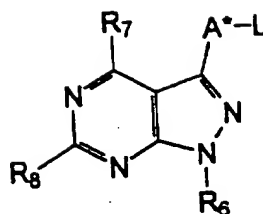
11 In these examples the nucleosides which are obtained in this  
12 scheme are incorporated into the desired SBC ODN, and the alkylating  
13 portion of the cross-linking agent is attached to the terminal amino  
14 group of "Y" only after removal of the respective phtalic or  
15 trifluoroacetyl blocking groups.

16 Another particularly preferred example of an "arm-leaving group  
17 combination" (A\*-L) is attachment of a nitrogen-mustard type alkylating  
18 agent (or other alkylating agent) to the amino function of a  
19 5-(3-aminopropyl)-2'-deoxyuridine building unit of the SBC ODN. The  
20 appropriate nucleotide building unit for ODN synthesis which includes  
21 the 5-(3-aminopropyl)-2'-deoxyuridine nucleoside moiety can be  
22 obtained in analogy to Reaction Scheme 1, and in accordance with the  
23 teaching of Meyer et al., J. Am. Chem. Soc. 1989, 111, 8517. In this  
24 particularly preferred embodiment the nucleotide having the  
25 5-(3-aminopropyl)-2'-deoxyuridine moiety is incorporated into the SBC  
26 ODN by routine synthesis, and the cross-linking function is introduced  
27 by reacting the SBC ODN with an activated form of a "nitrogen  
28 mustard", such as 2,3,5,6-tetrafluorophenyl-4'-[bis(2-chloroethyl)-  
29 amino]phenyl- butyrate (Chlorambucil 2,3,5,6-tetrafluorophenyl ester;  
30 chlorambucil itself is commercially available).



### Reaction Scheme 1

Other examples of nucleotides where the crosslinking agent is attached to a heterocyclic base, are 2'-deoxy-4-aminopyrazolo[3,4-d]pyrimidine derivatives. The general structure of these derivatives is shown below in Formula 14.  $A^*-L$  represents the "arm" and the "leaving group" of the cross-linking functionality, as described above.  $R_s$  represents the sugar moiety as described above, and  $R_1$  and  $R_2$  independently are H, OR, SR, NHOR,  $NH_2$  or  $NH(CH_2)_tNH_2$ , where R is H or  $C_{1-6}$  alkyl, t is 0 to 12. These compounds can be made from 3,4-disubstituted and 3,4,6-trisubstituted pyrazolo[3,4-d]pyrimidines, in accordance with the teaching of Kobayashi in Chem. Phar. Bull. 21:941-951 (1973) which is incorporated herein by reference.



Formula 14

Discussing still in general terms the structures of the cross-linking class of SBC ODNs of the present invention, it is noted that examination of double-stranded DNA by ball-and-stick models and high resolution computer graphics indicates that the 7-position of the purines and the 5-position of the pyrimidines lie in the major groove of the B-form duplex of double-stranded nucleic acids. These positions can be substituted with side chains of considerable bulk without interfering with the hybridization properties of the bases. These side arms may be introduced either by derivatization of dThd or dCyd, or by straightforward total synthesis of the heterocyclic base, followed by glycosylation. These modified nucleosides may be converted into the appropriate activated nucleotides for incorporation into oligonucleotides with an automated DNA synthesizer. With the pyrazolo[3,4-*d*]pyrimidines, which are analogs of adenine, the crosslinking arm is attached at the 3-position, which is equivalent to the 7-position of purine.

The crosslinking side chain (arm = A\*) should be of sufficient length to reach across the major groove from a purine 7- or 8-position, pyrimidine 5-position, pyrrolopyrimidine 5-position or pyrazolopyrimidine 3-position and react with the N-7 of a purine (preferably guanine) located above (on the oligomer 3'-side) the base pair containing the modified analog.

1 The crosslinking side chain (arm = A\*) holds the functional group away  
2 from the base when the base is paired with another within the  
3 double-stranded complex. As noted above, broadly the arm A\* should  
4 be equivalent in length to a normal alkyl chain of 2 to 50 carbons.  
5 Preferably, the arms include alkylene groups of 1 to 12 carbon atoms,  
6 alkenylene groups of 2 to 12 carbon atoms and 1 or 2 olefinic bonds,  
7 alkynylene groups of 2 to 12 carbon atoms and 1 or 2 acetylenic bonds,  
8 or such groups substituted at a terminal point with nucleophilic groups  
9 such as oxy, thio, amino or chemically blocked derivatives thereof (e.g.,  
10 trifluoroacetamido, phthalimido, CONR', NR'CO, and SO<sub>2</sub>NR', where  
11 R' = H or C<sub>1-6</sub>alkyl). Such functionalities, including aliphatic or  
12 aromatic amines, exhibit nucleophilic properties and are capable of  
13 serving as a point of attachment to such groups as

14 - (CH<sub>2</sub>)<sub>m</sub> - L,  
15 - CO - (CH<sub>2</sub>)<sub>m</sub> - (X\*)<sub>n</sub> - N(R<sub>1</sub>) - (CH<sub>2</sub>)<sub>p</sub> - L, and  
16 - CO - CH<sub>2</sub> - L

17 which are described above as components of exemplary cross-linking  
18 functional groups.

19 After the nucleoside or nucleotide unit which carries the  
20 crosslinking functionality A\*-L, or a suitable precursor thereof, (such as  
21 the - (CH<sub>2</sub>)<sub>q</sub> - NH<sub>2</sub> or - (CH<sub>2</sub>)<sub>q</sub> - Y\* group, where Y\* terminates with a  
22 nucleophilic group such as NH<sub>2</sub>) is prepared, further preparation of the  
23 modified oligonucleotides of the present invention can proceed in  
24 accordance with state-of-the-art. Thus, to prepare oligonucleotides,  
25 protective groups are introduced onto the nucleosides or nucleotides  
26 and the compounds are activated for use in the synthesis of  
27 oligonucleotides. The conversion to protected, activated forms follows  
28 the procedures as described for 2'-deoxynucleosides in detail in several  
29 reviews. See, Sonveaux, Bioorganic Chemistry, 14:274-325 (1986);  
30 Jones, in "Oligonucleotide Synthesis, a Practical Approach", M.J. Gait,

1 Ed., IRL Press, p. 23-34 (1984).

2 The activated nucleotides are incorporated into oligonucleotides  
3 in a manner analogous to that for DNA and RNA nucleotides, in that  
4 the correct nucleotides will be sequentially linked to form a chain of  
5 nucleotides which is complementary to a sequence of nucleotides in  
6 target DNA or RNA. The nucleotides may be incorporated either  
7 enzymatically or via chemical synthesis. The nucleotides may be  
8 converted to their 5'-O-dimethoxytrityl-3'-(N,N-diisopropyl)-  
9 phosphoramidite cyanoethyl ester derivatives, and incorporated into  
10 synthetic oligonucleotides following the procedures in "Oligonucleotide  
11 Synthesis: A Practical Approach", supra. The N-protecting groups are  
12 then removed, along with the other oligonucleotide blocking groups, by  
13 post-synthesis aminolysis, by procedures generally known in the art.

14 In a preferred embodiment, the activated nucleotides may be  
15 used directly on an automated DNA synthesizer according to the  
16 procedures and instructions of the particular synthesizer employed. The  
17 oligonucleotides may be prepared on the synthesizer using the standard  
18 commercial phosphoramidite or H-phosphonate chemistries. The  
19 foregoing description for preparing the SBC ODNs of the invention  
20 applies not only to the SBC ODNs which bear one or more cross linking  
21 agents, but also generally to all SBC ODNs of the invention. However,  
22 as it is described in detail below, 2-thiothymine containing SBC  
23 nucleotides (T analogs) are more sensitive to treatment with ammonia  
24 (or other nucleophiles) than other generally used components for  
25 sequential ODN synthesis on an automatic synthesizer. The preferred  
26 methods for incorporating these components into the SBC ODNs of the  
27 invention, and other chemical processes which differ from the normally  
28 routine processes of automatic ODN synthesis, are described below.

29 A moiety containing the leaving group, such as a haloacyl group  
30 (CO-CH<sub>2</sub>-L where L is halogen for example I) or - CO - (CH<sub>2</sub>)<sub>m</sub> - (X\*)<sub>n</sub> -

1 N(R<sub>1</sub>)-(CH<sub>2</sub>)<sub>p</sub>-L group (even more preferably a  
2 CO-(CH<sub>2</sub>)<sub>3</sub>-C<sub>6</sub>H<sub>4</sub>-N-[CH<sub>2</sub>CH<sub>2</sub>Cl]<sub>2</sub>) may be added to the aminoalkyl or  
3 like groups (-CH<sub>2</sub>)<sub>q</sub>-Y\*) following incorporation into oligonucleotides  
4 and removal of any blocking groups. For example, addition of an  
5 α-haloacetamide may be verified by a changed mobility of the modified  
6 compound on HPLC, corresponding to the removal of the positive  
7 charge of the amino group, and by subsequent readdition of a positive  
8 charge by reaction with 2-aminoethanethiol to give a derivative with  
9 reverse phase HPLC mobility similar to the original  
10 aminoalkyl-oligonucleotide.

11 In the situations where the cross linking agent (A\*-L moiety) is  
12 attached to the 3' or 5' terminus of the oligonucleotide, for example by  
13 an alkylamine linkage of the formula -(CH<sub>2</sub>)<sub>q</sub>-Y\* (Y\* terminating in an  
14 amine), the oligonucleotide synthesis may be performed to first yield the  
15 oligonucleotide with said aminoalkyl tail, to which then an alkylating  
16 moiety, such as the above-noted haloacylgroup (CO-CH<sub>2</sub>-L) or - CO -  
17 (CH<sub>2</sub>)<sub>m</sub> - (X\*)<sub>n</sub> - N(R<sub>1</sub>)-(CH<sub>2</sub>)<sub>p</sub>-L is introduced.

18 **SBC ODNs bearing a reporter group, lipophilic group or tail**

19 As is known in the art a "reporter group" can be broadly defined  
20 as a group that is incorporated in, or is attached to an ODN and which  
21 renders detection or isolation of the ODN possible by application of  
22 some analytical, physical, chemical or biochemical method. Generally  
23 speaking reporter groups are attached to ODNs when the ODNs are  
24 used as probes. In terms of attaching reporter groups to ODNs in the  
25 general sense, the art is well developed and is recited here only in a  
26 summary fashion. The SBC ODNs of the present invention having a  
27 reporter group (such as a radioactive label) attached, can be utilized  
28 substantially in accordance with state-of-the-art hybridization  
29 technology, to detect specific target sequences in duplex regions of  
30 nucleic acids. The advantage of the SBC ODNs of the present



1 invention, as compared to the prior art, is that the SBC ODN of the  
2 present invention can effectively invade and bind to the duplex nucleic  
3 acid sequence.

4 Thus, probes may be labeled by any one of several methods  
5 typically used in the art. A common method of detection is the use of  
6 autoradiography with  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^{32}\text{P}$  labeled probes or the like.  
7 Other reporter groups include ligands which bind to antibodies labeled  
8 with fluorophores, chemiluminescent agents, and enzymes.  
9 Alternatively, probes can be conjugated directly with labels such as  
10 fluorophores, chemiluminescent agents, enzymes and enzyme substrates.  
11 Alternatively, the same components may be indirectly bonded through a  
12 ligand-antiligand complex, such as antibodies reactive with a ligand  
13 conjugated with label. The choice of label depends on sensitivity  
14 required, ease of conjugation with the probe, stability requirements, and  
15 available instrumentation.

16 The choice of label dictates the manner in which the label is  
17 incorporated into the probe. Radioactive probes are typically made  
18 using commercially available nucleotides containing the desired  
19 radioactive isotope. The radioactive nucleotides can be incorporated  
20 into probes, for example, by using DNA synthesizers, by  
21 nick-translation, by tailing of radioactive bases in the 3' end of probes  
22 with terminal transferase or the 5'-end with a polynucleotide kinase.

23 Non-radioactive probes can be labeled directly with a signal (e.g.,  
24 fluorophore, chemiluminescent agent or enzyme) or labeled indirectly by  
25 conjugation with a ligand. For example, a ligand molecule is covalently  
26 bound to the probe. This ligand then binds to a receptor molecule  
27 which is either inherently detectable or covalently bound to a detectable  
28 signal, such as an enzyme or photoreactive compound. Ligands and  
29 antiligands may be varied widely. Where a ligand has a natural  
30 "antiligand", namely ligands such as biotin, thyroxine, and cortisol, it can  
31 be used in conjunction with its labeled, naturally occurring antiligand.

1 Alternatively, any haptenic or antigenic compound can be used in  
2 combination with a suitably labeled antibody. A preferred labeling  
3 method utilizes biotin-labeled analogs of oligonucleotides, as disclosed  
4 in Langer et al., Proc. Natl. Acad. Sci. USA 78:6633-6637 (1981), which  
5 is incorporated herein by reference.

6 Enzymes of interest as reporter groups will primarily be  
7 hydrolases, particularly phosphatases, esterases, ureases and  
8 glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent  
9 compounds include fluorescein and its derivatives, rhodamine and its  
10 derivatives, dansyl, umbelliferone, rare earths, etc. Chemiluminescers  
11 include luciferin, acridinium esters and 2,3-dihydrophthalazinediones,  
12 e.g., luminol. A further description of reporter groups and specific  
13 examples thereof can be found in United States Patent No. 5,419,966,  
14 the specification of which is expressly incorporated herein by reference.

15 The specific hybridization conditions are not critical and will vary  
16 in accordance with the investigator's preferences and needs. The  
17 particular hybridization technique is not essential to the invention.  
18 Hybridization techniques are generally described in "Nucleic Acid  
19 Hybridization, A Practical Approach", Hames and Higgins, Eds., IRL  
20 Press, 1985; Gall and Pardue, Proc. Natl. Acad. Sci., U.S.A., 63:378-383  
21 (1969); and John et al., Nature, 223:582-587 (1969). As improvements  
22 are made in hybridization techniques, they can readily be applied.

23 The amount of labeled probe which is present in the hybridization  
24 solution may vary widely. Generally, substantial excess of probe over  
25 the stoichiometric amount of the target duplex nucleic acid will be em-  
26 ployed to enhance the rate of binding of the probe to the target  
27 sequence.

28 After hybridization at a temperature and time period appropriate  
29 for the particular hybridization solution used, the glass, plastic, or filter  
30 support to which the probe-target hybrid is attached is introduced into a  
31 wash solution typically containing similar reagents as provided in the

1 hybridization solution. Either the hybridization or the wash medium  
2 can be stringent. After appropriate stringent washing, the correct  
3 hybridization complex may now be detected in accordance with the  
4 nature of the label.

5 The probe may be conjugated directly with the label. For  
6 example, where the label is radioactive, the support surface with  
7 associated hybridization complex substrate is exposed to X-ray film.  
8 Where the label is fluorescent, the sample is detected by first irradiating  
9 it with light of a particular wavelength. The sample absorbs this light  
10 and then emits light of a different wavelength which is picked up by a  
11 detector ("Physical Biochemistry", Freifelder, D., W. H. Freeman & Co.,  
12 1982, pp. 537-542). Where the label is an enzyme, the sample is  
13 detected by incubation with an appropriate substrate for the enzyme.  
14 The signal generated may be a colored precipitate, a colored or  
15 fluorescent soluble material, or photons generated by bioluminescence  
16 or chemiluminescence. The preferred label for dipstick assays generates  
17 a colored precipitate to indicate a positive reading. For example,  
18 alkaline phosphatase will dephosphorylate indoxyl phosphate which then  
19 will participate in a reduction reaction to convert tetrazolium salts to  
20 highly colored and insoluble formazans.

21 Detection of a hybridization complex may require the binding of  
22 a signal generating complex to a duplex of target and probe  
23 polynucleotides or nucleic acids. Typically, such binding occurs through  
24 ligand and antiligand interactions as between a ligand-conjugated probe  
25 and an antiligand conjugated with a signal.

26 The label may also allow indirect detection of the hybridization  
27 complex. For example, where the label is a hapten or antigen, the  
28 sample can be detected by using antibodies. In these systems, a signal is  
29 generated by attaching fluorescent or enzyme molecules to the  
30 antibodies or in some cases, by attachment to a radioactive label.  
31 (Tijssen, P., "Practice and Theory of Enzyme Immunoassays, Laboratory

1   Techniques in Biochemistry and Molecular Biology", Burdon, R.H., van  
2   Knippenberg, P.H., Eds., Elsevier, 1985, pp. 9-20).

3         The amount of labeled probe present in the hybridization solution  
4   may vary widely, depending upon the nature of the label, the amount of  
5   the labeled probe that can reasonably bind to the cellular target nucleic  
6   acids, and the precise stringency of the hybridization medium and/or  
7   wash medium. Generally, substantial probe excesses over the  
8   stoichiometric amount of the target will be employed to enhance the  
9   rate of binding of the probe to the target nucleic acids.

10        This aspect of the invention is also directed to a method for  
11   identifying target duplex nucleic acid sequences, which method  
12   comprises utilizing an SBC ODN probe including a label as described  
13   above.

14        In one embodiment, the method comprises the steps of:

- 15           (a) preparing nucleic acids in the sample to be tested;
- 16           (b) hybridizing to the target nucleic acids an SBC ODN  
17   probe wherein the SBC ODN is a matched pair where each ODN of the  
18   pair is complementary to one of the two complementary strands of the  
19   target nucleic acid sequence;
- 20           (c) washing the sample to remove unbound probe;
- 21           (d) incubating the sample with detecting agents; and
- 22           (e) inspecting the sample.

23        The above method may be conducted following procedures well  
24   known in the art.

25        The SBC ODNs of the present invention may also incorporate  
26   lipophilic groups, especially as a "tail" moiety attached to the 3' or 5'  
27   phosphate end of the ODN, and related tails, such as aminoalkyl groups  
28   (having approximately 3 to 20 carbons), or hydroxyalkyl groups (having  
29   approximately 3 to 20 carbons). As is known in the art, lipophilic  
30   groups are groups which due to their hydrophobic nature substantially

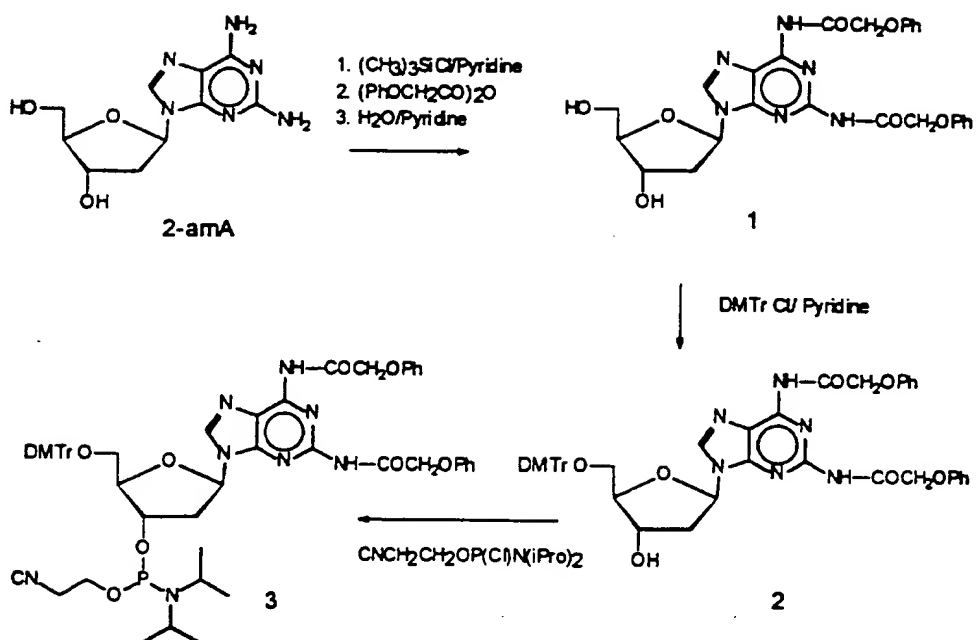
1 increase lipid solubility of a compound. Examples for lipophilic groups,  
2 are long chain (3 to 20 carbon alkyl, cycloalkyl groups, and compounds  
3 having a "steroid" skeleton such as cholesterol, cholic acid, progesterone  
4 and estradiol. Further examples of lipophilic groups are menthol and  
5 retinoic acid or analogs of retinoic acid. Synthetic methods suitable for  
6 attaching lipophilic and other tail moieties to the 3' or 5' end of the  
7 SBC ODNs of the present invention are described in United States  
8 Patent No. 5,419,966 the specification of which is expressly incorporated  
9 herein.

#### 10 Preparation of the SBC ODNs of the Invention

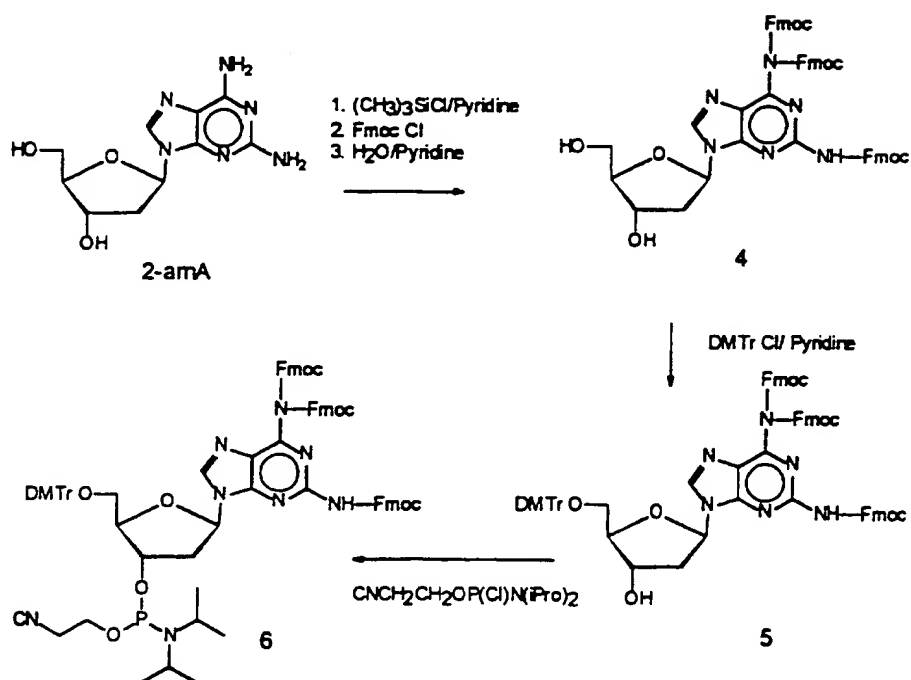
11 The nucleosides and nucleotides shown as components of the  
12 SBC ODNs of the present invention can be made by procedures known  
13 in the chemical literature. Oligonucleotide synthesis on an automatic  
14 synthesizer is generally described above in connection with the  
15 description of SBC ODNs containing a cross-linking functionality. A  
16 more detailed description of ODN synthesis with an automatic  
17 synthesizer utilizing a modified solid support, which is used in the  
18 currently preferred method for preparing the SBC ODNs of the  
19 invention, is described in United States Patent No. 5,419,966.

20 A modification of the standard "phosphoramidite" ODN synthesis  
21 procedure is used, however, when 2-thiothymine containing SBC ODNs  
22 are prepared because this heterocycle is more base labile than the  
23 natural base heterocycles of nucleic acids. Therefore, when this  
24 nucleotide is involved, milder treatment with ammonia is required in the  
25 step of removing blocking groups from the exocyclic amino groups of  
26 the nucleotide components and to remove the SBC ODN from the solid  
27 support. The modified procedures for preparing the suitably protected  
28 "phosphoramidite" reagents of 2,6-diaminopurine-2'-deoxyribofuranoside  
29 (Compounds 3 and 6) for nucleic acid synthesis are illustrated, in the  
30 alternative, in Reaction Schemes 2 and 3. As it can be seen in Reaction

1    **Scheme 2**, the phenoxyacetyl blocking group is attached to the exocyclic  
2    amino groups, whereas in **Reaction Scheme 3** the  
3    9-fluorenylmethoxycarbonyl (Fmoc) protecting group is used.  
4    N-phenoxyacetyl protected 2'-deoxyguanosine and 2'-deoxycytidine  
5    3'-O-2-cyanoethyl-N,N-diisopropylphosphoramidites are available  
6    commercially from BioGenex, Alameda, California.  
7    5'-O-Dimethoxytrityl-2-thiothymidine-3'-O-(2-cyanoethyl)-N,N-diisoprop  
8    ylphosphoramidite) can be obtained in accordance with the known  
9    literature procedure of Connolly et al. (1989) **Nucleic Acids Res.** **17**,  
10    4957-4974. 2,6-Diaminopurine-2'-deoxyriboside (the starting material in  
11    **Reaction Schemes 2 and 3**) can be obtained in accordance with the  
12    known literature procedure of Fathi et al. **Tetrahedron Lett.** **31**,  
13    319-322.



Reaction Scheme 2

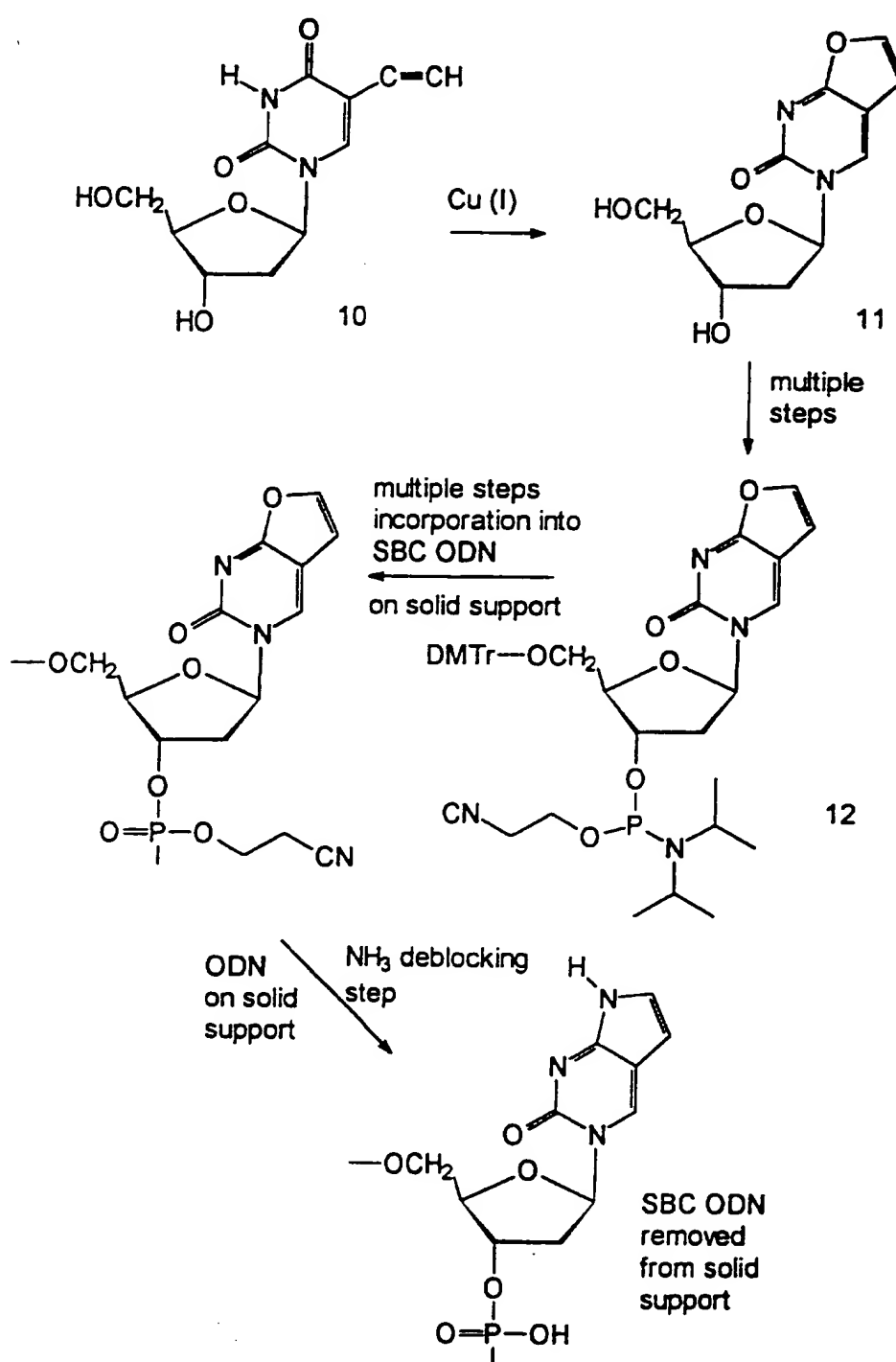


Reaction Scheme 3



1           The nucleotide moiety shown in Formula 4b is obtained by the  
2 method illustrated in Reaction Scheme 4 which substantially follows  
3 known chemical literature. First the "furan" analog deoxyribofuranoside,  
4 namely 3-(2'-deoxy- $\beta$ -D-ribofuranosyl)furano-[2,3-d]pyrimidine-  
5 6(5H)-one (Compound 11) is synthesized by copper (I)-catalyzed  
6 cyclization from the known antiviral nucleoside  
7 5-ethynyl-2'-deoxyuridine (Compound 10), substantially as in the  
8 literature procedure of Robins et al. J. Org. Chem. 1983, 48, 1854. This  
9 compound is dimethoxytritylated and converted into the corresponding  
10 cyanoethoxy phosphoramidite (Compound 12) suitable as a reagent for  
11 ODN synthesis, substantially by conventional literature methods (see  
12 Sinha et al. Nucleic Acids Research. 1984, 12, 4539). The SBC ODNs  
13 of the present invention are then constructed on a solid support. The  
14 final step of treating the SBC ODN with ammonia to remove protecting  
15 groups, converts the furano-[2,3-d]pyrimidine-6(5H)-one base into the  
16 pyrrolo-[2,3-d]pyrimidine-6(5H)-one base shown in Formula 4b. The  
17 Connolly et al. Nucleic Acids Res. 1989 17, 4957-4974, Fathi et al.  
18 Tetrahedron Lett. 1990 31, 319-322, Robins et al. J. Or. Chem. 1983, 48,  
19 1854 and Sinha et al. Nucleic Acid Research. 1984, 12, 4539 publications  
20 are expressly incorporated herein by reference.

31



### Reaction Scheme 4

**Use of the SBC ODNs of the invention and evidence of sequence specific selective binding ability**

Several oligonucleotides were prepared containing dI for dG, dP for dC, or containing d2-sT for dT and d2-amA for dA. The hybridization properties of these ODNs were studied by determining the melting temperature of the hybrids (under substantially physiological conditions) and by non-denaturing polyacrylamide gel electrophoresis (hereinafter PAGE) analysis. These measurements confirmed that each of the SBC ODNs forms a stable hybrid with the natural complementary or (substantially complementary) ODN, but not with the complementary SBC ODN. Thus, members of a matched pair of SBC ODNs were found to form stable hybrids with their respective natural complementary targets, but not with each other. Table 1 below indicates the melting temperatures observed under the conditions indicated in the table, and also the calculated decrease (drop) in melting temperature per modified base pair.

**Table 1**

**Table 1. T<sub>m</sub> Values for Native and Modified ODNs with dI and dP**

Watson: 5' XTY AXA AXY ATX YYA YYA XXY AAY YAY X 3'  
Crick: 3' YAX TYT TYX TAY XXT XXT YYX TTX XTX Y 5'

Hybrid	Watson		Crick		T <sub>m</sub> (°C) <sup>a</sup>	T <sub>m</sub> Drop per Modified Base Pair
	X	Y	X	Y		
I	C	G	C	G	75.6	0
II	P	I	C	G	48.2	1.61
III	C	G	P	I	57.2	1.08
IV	P	I	P	I	20.2	3.26

a: 10mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 50 mM NaCl, 10 mM MgCl<sub>2</sub>

1           In Table 1 the 28-mer ODN is a sequence taken from pBR 322  
2    plasmid. Hybrid 1 is formed from complementary  
3    oligodeoxynucleotides wherein X and Y are natural dC and dG residues  
4    in both ODNs. Thus, Hybrid 1 provides a reference, to which other  
5    hybrids formed of modified SBC ODNs can be compared. The pair of  
6    SBC ODNs shown as Hybrid IV in Table 1 comprises two 28-mer  
7    sequences where each of the natural dG and dC nucleotides is replaced  
8    with dI and dP, respectively. Hybrid IV is unstable with a melting  
9    temperature of 20.2° C. Nevertheless, each member of this pair forms a  
10   stable hybrid with its natural complement, in Hybrids II and III.

11           PAGE analysis also showed that the two members of the matched  
12   pair of SBC 28-mers do not hybridize in a stable manner, and that each  
13   SBC ODN and its natural complement form a stable hybrid. Moreover,  
14   the normal Watson strand showed no preference for the normal Crick  
15   strand over the SBC Crick strand because when equimolar amounts of  
16   these three strands were mixed simultaneously at room temperature  
17   about equal amounts of the duplex Hybrids I and III were formed.  
18   Additionally, there was little, if any, strand displacement or strand  
19   exchange when pre-formed Hybrid III was incubated with the normal  
20   homolog of the SBC strand, or with the Hybrid II. These data  
21   demonstrate that the SBC ODNs behave like natural ODNs when  
22   hybridized with their unmodified complementary strands, while they do  
23   not form stable hybrids with themselves.

24           Table 2 refers to a complementary pair of 20-mer  
25   oligodeoxyribonucleotides (ODN V and ODN VI) which are hybridized  
26   under substantially physiological conditions (0.2M NaCl, 0.01M  
27   Na<sub>2</sub>HPO<sub>4</sub>, 0.1mM EDTA, pH7.0, ODN concentration =  $4 \times 10^{-7}$ M).  
28   The ODNs designated in Table 2 as SBC(V) and SBC(VI) are modified  
29   so that each dA and each dT is replaced with the d2amA and d2sT,  
30   respectively. The melting temperatures of these pairs are indicated in

1 the Table.

2 **Table 2**

3 ODN V 5'-GTAAGAGAATTATGCAGTGC-3'

4 ODN VI 3'-CATTCTCTTAATACGTCACG-5'

5 SBC(V) 5'-G2sT2amA2amAG2amAG2amA2amA2sT2sT2amA2sTGC2-  
6 amAG2sTGC-3'

7 SBC(VI) 3'-C2amA2sT2sTC2sTC2sT2sT2amA2amA2sT2amACG2s-  
8 TC2amACG-5'

9  
10 **MELTING TEMPERATURE OF HYBRIDS**  
11 **ODN(V) ODN(VI) SBC(V) SBC(VI)**

12				
13	ODN(V)	-	55° C	- 64° C
14				
15	ODN(VI)	55° C	-	65° C -
16				
17	SBC(V)	-	65° C	- 26° C
18				
19	SBC(VI)	64° C	-	26° C -
20				

21 As it can be seen, the ODNs fully modified with the preferred A'  
22 and T' modifications of the present invention exhibit even stronger  
23 binding to the natural complementary ODNs than the binding between  
24 two natural complementary strands. At the same time, the matched  
25 pair of SBC ODNs are nevertheless incapable of forming a stable hybrid  
26 with each other (their melting temperature is 26°C).

27 Additional experiments conducted in accordance with the present  
28 invention, in terms of melting temperature measurements and PAGE  
29 analysis, showed that a matched pair of SBC ODNs complementary to  
30 both strands of a target sequence of double stranded DNA is capable of

1 invading the natural duplex nucleic acid to give a stable 3-armed joint.  
2 Analogous paired normal DNA ODNs failed to invade the same target.  
3 In case of long double stranded DNA, sequentially hybridizing the  
4 paired SBC ODNs to each member of the DNA target and then  
5 combining these hybrids results in stable double D loop formation which  
6 is stabilized by the bonding between each member of the SBC ODN  
7 pair and the corresponding complementary sequence in the target DNA.  
8 The resulting three-arm joints between the SBC ODNs and the DNA  
9 can be cleaved by resolvase enzymes. Strand invasion and double  
10 D-loop formation in long DNA by paired SBC ODNs is catalyzed by  
11 recombinase enzymes such as recA. Cleavage of these sites by resolvase  
12 will allow restriction of very long DNA, as from genomic DNA or  
13 cDNA libraries, at any pre-selected site. Therefore, the SBC ODNs of  
14 the invention can be used for gene mapping and like analytical and  
15 diagnostic purposes. The SBC ODNs can also be utilized to inhibit or  
16 block expression of a target gene, especially when one or preferably  
17 both members of the matched pair of SBC ODNs include a cross-linking  
18 function. In such a case, after double D loop formation with the target  
19 sequence of the gene, both strands of the nucleic acid are covalently  
20 linked to the SBC ODN, resulting in effective suppression of the gene.  
21 SBC ODNs having cross-linking functions can also be utilized for gene  
22 mapping and like diagnostic purposes.

23 Diagnostic and other "probe" like applications of the SBC ODNs  
24 of the invention also extend to messenger and ribosomal RNA, because  
25 a matched pair of SBC ODNs is able to sequence specifically invade the  
26 secondary structure of these duplex ribonucleic acids. Therapeutic use  
27 is in the anti-sense field, especially when the SBC ODN includes a  
28 cross-linking functionality. It is known in the art that the sequence of  
29 ribosomal RNA of bacteria is species specific. Furthermore, detection  
30 of this rRNA in DNA probe-based assays is usually hampered by lack of

1 access of the probe to the RNA because of secondary structure.  
2 Accordingly, SBC ODNs designed to sequence specifically invade  
3 bacterial ribosomal RNA are used, in accordance with the present  
4 invention, in diagnostic applications to diagnose bacterial infections in  
5 humans and animal species.

## 6 EXPERIMENTAL SECTION -- SPECIFIC EXAMPLES

### 7 Synthesis of pyrazolo[3,4-d]pyrimidin nucleotides

#### 8 EXAMPLE 1:

##### 9 6-(Tritylamino)caproic Acid.

10 6-Aminocaproic acid (26 g, 0.2 mole) was dissolved in  
11 dichloromethane (200 mL) by the addition of triethylamine (100 mL).  
12 Trityl chloride (120 g, 0.45 mole) was added and the solution stirred for  
13 36 hours. The resulting solution was extracted with 1N HCl and the  
14 organic layer evaporated to dryness. The residue was suspended in  
15 2-propanol/1N NaOH (300 mL/100 mL) and refluxed for 3 hours. The  
16 solution was evaporated to a thick syrup and added to dichloromethane  
17 (500 mL). Water was added and acidified. The phases were separated,  
18 and the organic layer dried over sodium sulfate and evaporated to  
19 dryness. The residue was suspended in hot 2-propanol, cooled, and  
20 filtered to give 43.5 (58%) of 6-(tritylamino)caproic acid, useful as an  
21 intermediate compound.

#### 22 EXAMPLE 2:

##### 23 5-(Tritylamino)pentylhydroxymethylenemalononitrile.

24 To a dichloromethane solution of 6-(tritylamino)-caproic acid  
25 (20.0 g, 53 mmole) and triethylamine (20 mL) in an ice bath was added  
26 dropwise over 30 min isobutylchloroformate (8.3 mL, 64 mmole). After  
27 the mixture was stirred for 2 hours in an ice bath, freshly distilled  
28 malononitrile (4.2 g, 64 mmole) was added all at once. The solution  
29 was stirred for 2 hours in an ice bath and for 2 hours at RT. The  
30 dichloromethane solution was washed with ice cold 2N HCl (300 mL)  
31 and the biphasic mixture was filtered to remove product that

1 precipitated (13.2 g). The phases were separated and the organic layer  
2 dried and evaporated to a thick syrup. The syrup was covered with  
3 dichloromethane and on standing deposited fine crystals of product.  
4 The crystals were filtered and dried to give 6.3 g for a total yield of 19.5  
5 g (87%) of the product, which is useful as an intermediate.

6 **EXAMPLE 3:**

7 5-(Tritylamino)pentylmethoxymethylenemalononitrile.

8 A suspension of the malononitrile of Example 2 (13 g, 31 mmole)  
9 in ether/dichloromethane (900 mL/100 mL), cooled in an ice bath, was  
10 treated with a freshly prepared ethereal solution of diazomethane (from  
11 50 mmole of Diazald<sup>R</sup> (Aldrich Chemical Company)). The solution was  
12 stirred for 6 hours and then neutralized with acetic acid (10 mL). The  
13 solution was evaporated to dryness and the residue chromatographed on  
14 silica gel using dichloromethane/acetone (4/1) as the eluent. Fractions  
15 containing product were pooled and evaporated to a syrup. The syrup  
16 was triturated with dichloromethane to induce crystallization. The  
17 crystals were filtered and dried to give 8.3 g (61%) of  
18 chromatographically pure product, useful as an intermediate compound.

19 **EXAMPLE 4:**

20 5-Amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile.

21 To a methanol solution (100mL) of the product of Example 3  
22 (7.0 g, 16 mmole) in an ice bath was added hydrazine monohydrate (7.8  
23 mL, 160 mmole) dropwise over 15 min. After stirring for 30 min in an  
24 ice bath, the solution was evaporated to dryness. The residue was  
25 suspended in cold methanol and filtered to give 7.1 g (100%) of  
26 5-amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile, useful as an  
27 intermediate, after drying. An analytical sample was prepared by  
28 recrystallization from water.

29 **EXAMPLE 5:**

30 5-Amino-1-(2-deoxy-3,5-di-O-toluoyl-β-D-erythropento-  
31 furanosyl)-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile.



1           An ice cold solution of the carbonitrile from Example 4 (3.5 g, 8  
2 mmole) was treated with sodium hydride and stirred for 30 min at  
3 0-4°C. 1-Chloro-1,2-dideoxy-3,5-di-O-toluoylribofuranose was added  
4 and the solution stirred for 1 hour at 0-4°C. The solution was poured  
5 into a saturated solution of sodium bicarbonate and extracted with  
6 dichloromethane. The organic layer was dried over sodium sulfate and  
7 evaporated to dryness. The residue was flash chromatographed. The  
8 organic layer was dried over sodium sulfate and evaporated to dryness.  
9 The residue was flash chromatographed on silica gel using toluene/ethyl  
10 acetate (5/1) as eluent. Two major products were isolated and  
11 identified as the N-1 and N-2 isomers in 57% (3.6 g) and 20% (1.2 g)  
12 N-1 and N-2 yields, respectively. Approximately 1 g of a mixture of N-1  
13 and N-2 isomers was also collected. Overall yield of glycosylated  
14 material was 5.8 g (92%). The N-1 isomer,  
15 5-amino-1-(2-deoxy-3,5-di-o-toluoyl-β-D-erythropentofuranosyl)-3-[(5-trit  
16 ylamino)-pentyl]pyrazole-4-carbonitrile, was used without further  
17 purification in Example 6.

18 **EXAMPLE 6:**

19 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trityl-  
20 amino)-pentyl]pyrazolo[3,4-d]pyrimidin-4-amine.

21           To a toluene (100 mL) solution of the pyrazole-4-carbonitrile of  
22 Example 5 (3.5 g, 4.4 mmole) was added diethoxymethyl acetate (1.1  
23 mL, 6.7 mmole). The solution was kept at 80-90°C for 5 hours and then  
24 evaporated to a syrup. The syrup was dissolved in dichloromethane (10  
25 mL) and added to ice cold methanolic ammonia (100 mL) in a glass  
26 pressure bottle. After two days at RT the contents of the bottle were  
27 evaporated to dryness. The residue was dissolved in methanol and  
28 adjusted to pH 8 with freshly prepared sodium methoxide to complete  
29 the deprotection. After stirring overnight the solution was treated with  
30 Dowex<sup>R</sup>-50 H+ resin, filtered and evaporated to dryness. The residue  
31 was chromatographed on silica gel using acetone/hexane (3/2) as eluent

1 to give 2.0 g (77%) of analytically pure product.

2 EXAMPLE 7:

3 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trityl-  
4 amino)-pentyl]pyrazolo[3,4-d]pyrimidin-4-amine 5'-monophosphate.

5 To an ice cold solution of the pyrazolopyrimidin-4-amine of  
6 Example 6 (250 mg, 0.43 mmole) in trimethyl phosphate (5 mL) was  
7 added phosphoryl chloride (50 μL) and the solution was kept at 0-4°C.  
8 The reaction was monitored by reversed phase HPLC using a linear  
9 gradient from 0 to 100% acetonitrile in water over 25 min. After  
10 stirring for 5 hours, an additional aliquot of phosphoryl chloride (25 μL)  
11 was added and the solution was stirred another 30 min. The solution  
12 was poured into 0.1M ammonium bicarbonate and kept in the cold  
13 overnight. The solution was then extracted with ether and the aqueous  
14 layer evaporated to dryness. The residue was dissolved in water (5 mL)  
15 and purified by reversed phase HPLC using a 22mm X 50cm C18  
16 column. The column was equilibrated in water and eluted with a  
17 gradient of 0 to 100% acetonitrile over 20 min. Fractions containing  
18 the desired material were pooled and lyophilized to give 160 mg (56%)  
19 of chromatographically pure nucleotide.

20 EXAMPLE 8:

21 1-(2-Deoxy-β-D-erythropentofuranosyl)-3-{5-[(6-bio-  
22 tinamido)hexanamido]pentyl}pyrazolo[3,4-d]pyrimidin-4-amine  
23 5'-monophosphate.

24 An ethanol solution (10 mL) of the nucleotide of Example 7,  
25 palladium hydroxide on carbon (50 mg), and cyclohexadiene (1 mL) was  
26 refluxed for 3 days, filtered, and evaporated to dryness. The residue  
27 was washed with dichloromethane, dissolved in DMF (1.5 mL)  
28 containing triethylamine (100 mL), and treated with  
29 N-hydroxy-succinimidyl biotinylaminocaproate (50 mg). After stirring  
30 overnight an additional amount of N-hydroxysuccinimidyl  
31 6-biotinamidocaproate (50 mg) was added and the solution was stirred

1 for 18 hours. The reaction mixture was evaporated to dryness and  
2 chromatographed following the procedure in Example 7. Fractions were  
3 pooled and lyophilized to give 80 mg of chromatographically pure  
4 biotinamido-substituted nucleotide.

5 EXAMPLE 9:

6 1-(2-Deoxy- $\beta$ -D-erythropentofuranosyl)-3-[5-(6-biotin-  
7 amido)-hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-4-amine  
8 5'-triphosphate.

9 The monophosphate of Example 8 (80 mg, ca. 0.1 mmole) was  
10 dissolved in DMF with the addition of triethylamine (14  $\mu$ L).  
11 Carbonyldiimidazole (81 mg, 0.5 mmole) was added and the solution  
12 stirred at RT for 18 hours. The solution was treated with methanol (40  
13  $\mu$ L), and after stirring for 30 minutes tributylammonium pyrophosphate  
14 (0.5 g in 0.5 mL DMF) was added. After stirring for 24 hours another  
15 aliquot of tributylammonium pyrophosphate was added and the solution  
16 was stirred overnight. The reaction mixture was evaporated to dryness  
17 and chromatographed following the procedure in Example 8. Two  
18 products were collected and were each separately treated with conc.  
19 ammonium hydroxide (1 mL) for 18 hours at 55°C. UV and HPLC  
20 analysis indicated that both products were identical after ammonia  
21 treatment and were pooled and lyophilized to give 35.2 mg of  
22 nucleoside triphosphate.

23 EXAMPLE 10:

24 NICK-TRANSLATION REACTION

25 The triphosphate of Example 9 was incorporated into pHPV-16  
26 using the nick translation protocol of Langer et al. (supra). The probe  
27 prepared with the triphosphate of Example 9 was compared with probe  
28 prepared using commercially available bio-11-dUTP (Sigma Chemical  
29 Co). No significant differences could be observed in both a filter  
30 hybridization and in in situ smears.

31 More specifically, the procedure involved the following materials

1 and steps

2 Materials:

3 DNase (ICN Biomedicals) - 4 $\mu$ g/mL

4 DNA polymerase 1 (U.S. Biochemicals) -

5 8 U/mL

6 pHPV - 16 - 2.16 mg/mL which is a

7 plasmid containing the genomic

8 sequence of human papillomavirus

9 type 16.

10 10X-DP - 1M Tris, pH 7.5 (20 mL); 0.5M

11 OTT (80 mL); 1M MgCl<sub>2</sub> (2.8 mL);

12 H<sub>2</sub>O (17 mL)

13 Nucleotides - Mix A - 2mM each dGTP,

14 dCTP, TTP (Pharmacia)

15 Mix U - 2mM each dGTP, dCTP,

16 dATP

17 Bio-11-dUTP - 1.0 mg/mL (BRL)

18 Bio-12-dAPPTP - 1.0 mg/mL

19 Steps:

20 To an ice cold mixture of 10X-DP (4 mL), pHPV-16 (2 mL),  
21 nucleotide mix A (6 mL), Bio-12-dAPPTP (2 mL), and H<sub>2</sub>O (20 mL)  
22 was added DNase (1 mL) and DNA polymerase 1 (2.4 mL). The  
23 reaction mixture was incubated at 16°C for 1 hour. The procedure was  
24 repeated using Bio-11-dUTP and nucleotide mix U in place of  
25 Bio-12-dAPPTP (comprising the triphosphate of Example 9) and  
26 nucleotide mix A.

27 Nucleic acid was isolated by ethanol precipitation and hybridized  
28 to pHPV-16 slotted onto nitrocellulose. The hybridized biotinylated  
29 probe was visualized by a streptavidin-alkaline phosphatase conjugate  
30 with BCIP/NBT substrate. Probe prepared using either biotinylated  
31 nucleotide gave identical signals. The probes were also tested in an in

1 situ format on cervical smears and showed no qualitative differences in  
2 signal and background.

3 **EXAMPLE 11:**

4 5-Amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbox- amide.

5 Following the procedure of Example 2, except that  
6 cyanoacetamide is used instead of malononitrile,  
7 5-(tritylamino)pentylhydroxymethylececyanoacetamide is prepared from  
8 6-(tritylamino)caproic acid. This is then treated with diazomethane to  
9 give the methoxy derivative, following the procedures of Example 3,  
10 which is then reacted with hydrazine monohydrate, as in Example 4, to  
11 give 5-amino-3-[(5-tritylamino)-pentyl]pyrazole-4-carboxamide.

12 **EXAMPLE 12:**

13 4-Hydroxy-6-methylthio-3-[(5-tritylamino)pentyl]pyra-  
14 zolo-[3,4-d]pyrimidine.

15 The carboxamide from Example 11 is reacted with potassium  
16 ethyl xanthate and ethanol at an elevated temperature to give the  
17 potassium salt of 4-hydroxypyrazolo[3,4-d]pyrimidine-6-thiol. This salt is  
18 then reacted with iodomethane to give 4-hydroxy-6-methylthio-3-  
19 [(5-tritylamino)pentyl]pyrazolo[3,4-d]pyrimidine.

20 **EXAMPLE 13:**

21 1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-  
22 (tritylamino)pentyl]pyrazolo[3,4-d]pyrimidin-6-amine.

23 Following the procedure of Example 5, the pyrazolopyrimidine of  
24 Example 12 is treated with sodium hydride and reacted with  
25 1-chloro-1,2-dideoxy-3,5-di-O-toluoylribofuranose. The resulting  
26 compound is reacted with MCPBA and with methanolic ammonia, and  
27 the toluoyl protecting groups are removed to give the product.

28 **EXAMPLE 14:**

29 1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-  
30 (6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin- 6-amine  
31 5'-monophosphate.

1       Following the procedure of Example 7, the pyrazolopyrimidine of  
2       Example 13 is reacted with phosphoryl chloride to give the  
3       corresponding 5'-monophosphate.

4       Following the procedure of Example 8, the above  
5       5'-monophosphate is reacted with palladium/carbon and cyclohexadiene,  
6       and the residue is reacted with N-hydroxy-succinimidyl  
7       biotinylaminocaproate to give  
8       1-(2-deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-  
9       (6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-6-amine  
10      5'-monophosphate.

11      EXAMPLE 15:

12      1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-  
13      (6-biotinamido)hexanamidopentyl]pyrazolo[3,4-d]pyrimidin-6-amine  
14      5'-triphosphate.

15      Following the procedure of Example 9, the 5'-monophosphate of  
16      Example 14 is treated with carbonyldiimidazole and then reacted with  
17      tributylammonium pyrophosphate to give the corresponding  
18      5'-triphosphate.

19      EXAMPLE 16:

20      1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trityl-  
21      amino)-pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoylamine.

22      1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(tri-  
23      tylamino)pentyl]pyrazolo[3,4-d]pyrimidine-4-amine from Example 6 is  
24      reacted with benzoyl chloride and pyridine to give  
25      1-(2-deoxy-3,5-di-O-benzoyl-β-D-erythropentofuranosyl)-3-[5-(tritylamino  
26      )pentyl]pyrazolo- [3,4-d]-pyrimidine-4-dibenzoylamine. This is treated  
27      with aqueous sodium hydroxide to partially deprotect the compound  
28      giving 1-(2-deoxy-β-D-erythropentofuranosyl)-3-[5-(tritylamino)-  
29      pentyl]pyra- zolo[3,4-d]pyrimidine-4-benzoylamine.

30      EXAMPLE 17:

31      1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trifluoro-

1 acetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl- amine.

2       Following the procedure of Example 8, the benzoylamine of  
3 Example 16 is treated with palladium hydroxide on carbon and then  
4 with trifluoroacetic anhydride to give 1-(2-deoxy-β-D-erythropentofuran-  
5 osyl)-3-[5-(trifluoroacetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl  
6 amine.

7 EXAMPLE 18:

8 1-(2-Deoxy-5-O-dimethoxytrityl-β-D-erythropentofuran-  
9 osyl)-3-[5-(trifluoroacetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoyl  
10 amine 3'-O-(N,N-diisopropyl)phosphoramidite cyanoethyl ester.

11       The compound of Example 17 is reacted with dimethoxytrityl  
12 chloride and pyridine to give the corresponding 5'-dimethoxytrityl  
13 compound. This compound is then reacted with cyanoethyl  
14 chloro-N,N-diisopropylphosphoramidite (according to the method of  
15 Sinha et al., Nucleic Acids Res., 12:4539 (1984)) to give the  
16 3'-O-activated nucleoside.

17 Synthesis of nucleotides and ODNs including a cross-linking function

18 EXAMPLE 19

19 5-(4-Phthalimidobut-1-yn-1-yl)-2'-deoxyuridine

20       5-Iodo-2'-deoxyuridine (354 mg, 1 mmol) was dissolved in 10 mL  
21 of dimethylformamide. Cuprous iodide (76 mg, 0.4 mmol),  
22 tetrakis(triphenylphosphine)palladium(0) (230 mg, 0.2 mmol), and  
23 triethylamine (200 mg, 2.0 mmol) were added. 4-Phthalimidobut-1-yne  
24 (300 mg, 1.5 mmol) was added all at once and the reaction kept at 60°C  
25 for three hours. The clear yellow reaction was then evaporated and  
26 methylene chloride was added. Scratching of the flask induced  
27 crystallization of nearly all of the product which was filtered and  
28 recrystallized from 95% ethanol to give 335 mg (78%) of title  
29 compound as fine, feathery needles.

30 EXAMPLE 20

31 5-(4-Phthalimidobut-1-yl)-2'-deoxyuridine

1           1.00 Gram of 5-(4-Phthalimidobut-1-yn-1-yl)-2'-deoxyuridine was  
2 dissolved in 95% EtOH and about 3 g of neutral Raney nickel was  
3 added. After 48 hours, the catalyst was removed by cautious filtration  
4 and the filtrate was evaporated to a solid which was recrystallized from  
5 methanol-water to give 960 mg (97%) of the title compound.

6 **EXAMPLE 21:**

7 **5-(3-Iodoacetamidopropyl)-2'-deoxyuridine.**

8           5-(3-Trifluoroacetamidoprop-1-yl)-2'-deoxyuridine (0.3 mmol) is  
9 treated with ammonia and then with N-hydroxy-succinimidyl  
10  $\alpha$ -iodoacetate (0.5 mmol). The reaction mixture is evaporated to  
11 dryness and purified by chromatography to give  
12 5-(3-iodoacetamidopropyl)-2'-deoxyuridine.

13 **EXAMPLE 22 5-(4-(4-Bromobutyramido)butyl)-2'-deoxyuridine**

14           5-(4-phthalimidobut-1-yl)-2'-deoxyuridine is treated with ammonia  
15 and then with N-hydroxysuccinimidyl-4-bromobutyrate to give  
16 5-(4-(4-bromobutyramido)butyl)-2'-deoxyuridine.

17 **Preparation of Synthetic Oligonucleotides**

18 **EXAMPLE 23:**

19 **Phosphoramidite Preparation and DNA Synthesis.**

20           Nucleosides were 5'-dimethoxytritylated, following known  
21 procedures, to give around 85% yield, and the 3'-phosphoramidite was  
22 made using diisopropylamino  $\beta$ -cyanoethylchlorophosphite (as described  
23 in "Oligonucleotide Synthesis: A Practical Approach", supra) with  
24 diisopropylethylamine in methylene chloride. The phosphoramidite was  
25 made into a 0.2N solution in acetonitrile and placed on the automated  
26 DNA synthesizer. Incorporation of these new and modified  
27 phosphoramidites gave incorporation similar to ordinary  
28 phosphoramidites (97-99% as judged by assay of the trityl color released  
29 by UV.)

30           Oligonucleotides were removed from the DNA synthesizer in  
31 tritylated form and deblocked using 30% ammonia at 55°C for 6 hours.



1 Ten  $\mu\text{L}$  of 0.5M sodium bicarbonate was added to prevent acidification  
2 during concentration. The oligonucleotide was evaporated to dryness  
3 under vacuum and redissolved in 1.0 mL water. The oligonucleotides  
4 were purified by HPLC using 15-55% acetonitrile in 0.1N  
5 triethylammonium acetate over 20 minutes. Unsubstituted  
6 oligonucleotides came off at 10 minutes; amino derivatives took 11-12  
7 minutes. The desired oligonucleotide was collected and evaporated to  
8 dryness, then it was redissolved in 80% aqueous acetic acid for 90  
9 minutes to remove the trityl group. Desalting was accomplished with a  
10 G25 Sephadex column and appropriate fractions were taken. The  
11 fractions were concentrated, brought to a specific volume, dilution  
12 reading taken to ascertain overall yield and an analytical HPLC done to  
13 assure purity. Oligonucleotides were frozen at  $-20^{\circ}\text{C}$  until use.

14 In general, to add the crosslinking arm to an  
15 aminoalkyloligonucleotide, a solution of 10  $\mu\text{g}$  of the  
16 aminoalkyloligonucleotide and a 100X molar excess of  
17 n-hydroxysuccinimide haloacylate such as  $\alpha$ -haloacetate or  
18 4-halobutyrate in 10  $\mu\text{L}$  of 0.1 M borate buffer, pH 8.5, is incubated at  
19 ambient temperature for 30 min. in the dark. The entire reaction is  
20 passed over a NAP-10 column equilibrated with and eluted with distilled  
21 water. Appropriate fractions based on UV absorbance are combined  
22 and the concentration is determined spectrophotometrically.

23 2,3,5,6-Tetrafluorophenyl trifluoroacetate.

24 A mixture of 2,3,5,6-tetrafluorophenol (55.2 g, 0.33 mol),  
25 trifluoroacetic anhydride (60 mL, 0.42 mol) and boron trifluoride  
26 etherate (0.5 mL) was refluxed for 16 hr. Trifluoroacetic anhydride and  
27 trifluoroacetic acid were removed by distillation at atmospheric  
28 pressure. The trifluoroacetic anhydride fraction (bp  $40^{\circ}\text{C}$ ) was returned  
29 to the reaction mixture along with 0.5 mL of boron trifluoride etherate,  
30 and the mixture was refluxed for 24 hr. This process was repeated two  
31 times to ensure complete reaction. After distillation at atmospheric

1 pressure, the desired product was collected at 62°C/45 mm (45°C/18  
2 mm) as a colorless liquid: yield = 81.3 g (93%); d = 1.52 g/mL;  $n_D^{21}$  =  
3 1.3747; IR (CHCl<sub>3</sub>) 3010, 1815, 1525, 1485, 1235, 1180, 1110, and 955  
4 cm<sup>-1</sup>. Anal. Calcd for C<sub>8</sub>HF<sub>7</sub>O<sub>2</sub>: C, 36.66; H, 0.38; F, 50.74. Found: C,  
5 36.31; H, 0.43; F, 50.95.

6 2,3,5,6-Tetrafluorophenyl-4'-[bis(2-chloroethyl)amino]phenylbutyrate  
7 (Chlorambucil 2,3,5,6-tetrafluorophenyl ester)

8 To a solution of 0.25 g (0.82 mmol) of chlorambucil (supplied by  
9 Fluka A. G.) and 0.3 g (1.1 mmol) of 2,3,5,6-tetrafluorophenyl  
10 trifluoroacetate in 5 ml of dry dichloromethane was added 0.2 ml of dry  
11 triethylamine. The mixture was stirred under argon at room temperature  
12 for 0.5 h and evaporated. The residual oil was purified by column  
13 chromatography on silica gel with hexane-chloroform (2:1) as the eluting  
14 solvent to give the ester as an oil: 0.28 g (75%); TLC on silica gel  
15 (CHCl<sub>3</sub>) R<sub>f</sub> 0.6; IR (in CHCl<sub>3</sub>) 3010, 1780, 1613, 1521, 1485 cm<sup>-1</sup>.

16 2-Propargyloxyethyl)amine (John, R., and Seitz, G., Chem. Ber., 123,  
17 133 (1990) was prepared by condensing propynol with  
18 2-bromoethylammonium bromide in liquid ammonia in the presence of  
19 Na NH<sub>2</sub>, and was used crude for the next reaction.

20 3-(2-Trifluoroacetamidoethoxy)propyne

21 (2-Propargyloxyethyl)amine (13.8 g, 0.14 mol) is stirred and  
22 chilled in an iso-propanol-dry ice bath while excess of trifluoroacetic  
23 anhydride (26 ml, 0.18 mol) is added dropwise.

24 N-(2-Propargyloxyethyl)trifluoroacetamide is distilled at 84-85°/1.7 torr  
25 as an oil which solidified upon refrigeration; yield 14.4 g (52%), m.p.  
26 (16°,  $n_D^{24}$  1.4110. Anal. Calcd. for C<sub>7</sub>H<sub>8</sub>F<sub>3</sub>NO<sub>2</sub>: C, 43.09, H, 4.13; N,  
27 7.18; F, 29.21. Found: C, 42.80; H, 4.03; N, 7.06; F, 29.38.

28 5-[3-(2-Trifluoroacetamidoethoxy)propynyl]-2'-deoxyuridine A

29 mixture of 5-iodo-2'-deoxyuridine (3.54 g, 10 mmol), copper(I) iodide  
30 (0.19 g, 1 mmol) and tetrakis(triphenylphosphine)palladium(0) (0.58 g,  
31 0.5 mmol) is dried in vacuo at 60° for 3 hours and placed under argon.

1 A suspension of the mixture in dry DMF (20 ml) is stirred under argon  
2 and treated with dry triethylamine (1.7 ml, 12 mmol) followed by  
3 3-(2-Trifluoroacetamidoethoxy)propyne (3.17 g, 16 mmol). The mixture  
4 is cooled at room temperature in a water bath and stirred for 17 hours.  
5 The mixture is treated with 2% acetic acid (100 ml), the catalyst is  
6 removed by filtration and washed with 50% methanol. The filtrates are  
7 combined and passed onto a LiChroprep RP-18 column (5X25 cm), the  
8 column is washed, then eluted with 1% acetic acid in 50% (v/v)  
9 methanol. The fractions with the main product are combined,  
10 evaporated, and dried in vacuo. The resultant foam is stirred with 150  
11 ml of ether to give crystalline product; yield 3.6 g (85%); m.p. 145-152°.  
12 5-[3-(2-Trifluoroacetamidoethoxy)propyl]2'-deoxyuridine

13 A solution of 5-[3-(2-trifluoroacetamidoethoxy)-  
14 propynyl]-2'-deoxyuridine (3.4 g, 8.1 mmol) in methanol (20 ml) is  
15 stirred with ammonium formate (prepared by addition of 3 ml, 79 mmol  
16 of cold 98% formic acid into 2 ml, 50 mmol of dry ice frozen 25%  
17 ammonia) and 0.2 g of 10% Pd/C for 7 hours at room temperature  
18 under hydrogen atmosphere. The catalyst is removed by filtration, the  
19 filtrate evaporated and product is purified on LiChroprep RP-18 column  
20 by the above procedure. Fractions containing the desired product are  
21 combined and evaporated to dryness in vacuo and the resultant solid is  
22 triturated with dry ether to give 3.0 g (87% product, m.p. 107-110°; <sub>max</sub>  
23 in nm, in 0.1M triethylamine-acetate (pH 7.5), 220, 268. Analysis  
24 calculated for C<sub>16</sub>H<sub>22</sub>F<sub>3</sub>N<sub>3</sub>O<sub>7</sub>: C, 45.18; H, 5.21; N, 9.88; F, 13.40. Found  
25 C, 45.16; H, 5.16; N, 9.68; F, 13.13.

26 Introduction of chlorambucil residue into the primary amino groups of  
27 oligonucleotides

28 Preparation of the cetyltrimethylammonium salt of oligonucleotides: a  
29 100 µL aliquot of aqueous solution of oligonucleotide (50-500 ug),  
30 generally triethylammonium salt, was injected to a column packed with  
31 Dowex 50wx8 in the cetyltrimethylammonium form and prewashed with

1 50% alcohol in water. The column was eluted by 50% aqueous ethanol  
2 (0.1 mL/min). Oligonucleotide containing fraction was dried on a  
3 Speedvac over 2 hours and used in following reactions.

4 Ethanol solution (50 uL) of cetyltrimethylammonium salt of an  
5 oligonucleotide (50-100  $\mu$ g) was mixed with 0.08 M solution of  
6 2,3,5,6-tetrafluorophenyl-4'-[bis(2-chloroethyl)amino]phenylbutyrate  
7 (tetrafluorophenyl ester of chlorambucil) in acetonitrile (50  $\mu$ L) and 3  
8  $\mu$ L of diisopropylethylamine. After shaking for three hours at room  
9 temperature, the product was precipitated by 2% LiClO<sub>4</sub> in acetone (1.5  
10 mL). The product was reprecipitated from water (60 uL) by 2% LiClO<sub>4</sub>  
11 in acetone three times. Finally the chlorambucil derivative of the  
12 oligonucleotide was purified by Reverse Phase Chromatography with  
13 approximately 50-80% yield. The fraction containing the product was  
14 concentrated by addition of butanol. The isolated chlorambucil  
15 derivative of the oligonucleotide was precipitated in acetone solution  
16 with LiClO<sub>4</sub>, washed by acetone and dried under vacuum. All  
17 manipulations of reactive oligonucleotide were performed as quickly as  
18 possible, with the product in ice-cold solution.

19 **Preparation of SBC ODNs**

20 N-phenoxyacetyl protected 2'-deoxyguanosine and 2'-deoxycytidine  
21 3'-O-2-cyanoethyl-N,N'-diisopropylphosphoramidites are available  
22 commercially from BioGenex, Alameda, California.

23 5'-O-dimethoxytrityl-2-thiothymi-  
24 dine-3'-O-(2-cyanoethyl-N,N'-diisopropylphosphoramidite) was prepared  
25 using the procedure of Connolly et al. supra.

26 2,6-diaminopurine-2'-deoxyriboside was synthesized as described by  
27 Fathi et al. supra.

28 **N<sup>2</sup>,N<sup>6</sup>-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deoxyriboside**  
29 **(Compound 1, Reaction Scheme 1).**

30 This compound is prepared substantially in accordance with the  
31 literature procedure of Schulhof et al.(1987) Nucleic Acids Res. 15,

1 397-416. 2,6-Diaminopurine-2'-deoxyribose (1.8 g, 6.8 mmol) is dried  
2 by evaporation with dry pyridine. Trimethylchlorosilane (5 mL, 39  
3 mmol) is added dropwise to an ice cold solution of  
4 2,6-diaminopurine-2'-deoxyribose in 35 mL of dry pyridine. After 30  
5 min, phenoxyacetic anhydride (8.0 g, 28 mmol) is added to the stirred  
6 solution. The mixture is kept for 3 h at RT then cooled to 5°C. Water  
7 (5 mL) is added to quench the excess of phenoxyacetic anhydride. After  
8 being stirred for 2 h, the reaction mixture is concentrated on a rotary  
9 evaporator to approximately 10 mL and then diluted with water to 120  
10 mL to give an emulsion. The emulsion is washed with ether (150 mL).  
11 The resulting precipitate is filtered, washed with ether, water, and dried  
12 in vacuo. The material (3.2 g, 87 %) obtained by using this procedure is  
13 pure enough to be used in the next step without additional purification.  
14 5'-O-Dimethoxytrityl-N<sup>2</sup>,N<sup>6</sup>-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deox  
15 yribose (Compound 2).

16 N<sup>2</sup>,N<sup>6</sup>-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deoxyribose (3.2  
17 g, 5.8 mmol) is dried by evaporation with dry pyridine (2x20 mL) and  
18 dissolved in 30 mL of the same solvent. 4,4'-dimethoxytrityl chloride  
19 (2.0 g, 6 mmol) is added in one portion with vigorous stirring. After 1 h,  
20 TLC (CHCl<sub>3</sub>/MeOH, 19:1 v/v) indicates complete reaction. The  
21 reaction mixture is concentrated on a rotary evaporator and diluted with  
22 dichloromethane to approximately 200 mL. After being washed with  
23 saturated NaHCO<sub>3</sub> (2x200 mL), the organic layer is dried with Na<sub>2</sub>SO<sub>4</sub>,  
24 and then concentrated in vacuo to an oil. Preparative silica gel  
25 chromatography with a gradient of MeOH in CH<sub>2</sub>Cl<sub>2</sub> from 0 to 5%  
26 provides the desired product as a crystalline solid (3.3 g, 68%).  
27 5'-O-Dimethoxytrityl-N<sup>2</sup>,N<sup>6</sup>-bis(phenoxyacetyl)-2,6-diaminopurine-2'-deox  
28 yribose-3'-O-(2-cyanoethyl-N,N'-diisopropylphosphoramidite)  
29 (Compound 3).

30 A suspension of Compound 2 (3.1 g, 3.7 mmol) in a mixture of

1 dichloromethane (30 mL) and diisopropylethylamine (4 mL) is treated  
2 with 2-cyanoethoxy N,N-diisopropylaminochlorophosphine (1.6 mL, 7.2  
3 mmol). The reaction is stirred for 1 h, and quenched by addition of  
4 methanol (0.1 mL). After 2 min, dichloromethane (70 mL) is added and  
5 the solution is washed with 1 M NaHCO<sub>3</sub> (100 mL) followed by  
6 saturated brine (100 mL). The organic layer is dried, filtered, and the  
7 solvent is removed in vacuo. The crude product is purified by  
8 preparative silica gel chromatography (ethyl  
9 acetate/dichloromethane/triethylamine, 45:45:5 v/v/v). After the  
10 purification the product is additionally precipitated in hexane to give a  
11 colorless solid (2.5 g, 65 %).

12 N,N,N'-tris(9-fluorenylmethoxycarbonyl)-2,6-diaminopu-  
13 rine-2'-deoxyriboside (Compound 4, Reaction Scheme 2)

14 2,6-Diaminopurine-2'-deoxyriboside (2.3 g, 8.5 mmol) is dried by  
15 evaporation with dry pyridine and dissolved in 40 mL of the same  
16 solvent. Trimethylchlorosilane (5 mL, 3.9 mmol) is added dropwise to  
17 the ice cold solution and the reaction is kept for 1 min at 5°C and 15  
18 min at RT. 9-Fluorenylmethoxy carbonyl chloride (6.2 g, 24 mmol) is  
19 added, and the reaction mixture is stirred for 2 h. Hydrolysis of the  
20 trimethylsilyl groups and of excess chlorides is effected by addition of  
21 water (30 mL). After stirring for 18 h, the mixture is evaporated to near  
22 dryness and co-evaporated with toluene to remove residual pyridine.  
23 Upon addition of water (150 mL) a white solid is precipitated. The  
24 suspension is shaken with ether (100 mL) and is then filtered to give an  
25 off-white solid. TLC (CHCl<sub>3</sub>/MeOH, 9:1 v/v) shows at least three new  
26 products. The major product with higher R<sub>f</sub> is isolated by silica gel  
27 chromatography using a gradient of methanol in dichloromethane. The  
28 product is a white solid (1.2 g, 15%).

29 5'-O-Dimethoxytrityl-N,N,N'-tris(9-fluorenylmethoxycarbonyl)-2,6-diamin  
30 opurine-2'-deoxyriboside (Compound 5).

31 The title compound is prepared in accordance with the procedure

described for the phenoxyacetylated analog in 70% yield.

5'-O-Dimethoxytrityl-N,N,N'-tris(9-fluorenylmethoxycarbonyl)-2,6-diaminopurine-2'-deoxyriboside-3'-O-(2-cyanoethyl-N,N'-diisopropylphosphoramidite) (Compound 6).

The general method demonstrated described for the phenoxyacetylated analog **Compound 3** (see above) is used to synthesize this phosphoramidite.

#### Preparation of hexanol-oxalyl Primer Support

This support is made by analogy to the literature method (Alul et al. Nucleic Acids Res. (1991) 19, 1527-1532). Solution I is prepared by dissolving of 2.8 g (6.7 mmol) of O-(4,4'-dimethoxytrityl)-1,6-hexanediol (5) in dry acetonitrile (8 mL). To prepare solution II, oxalyl chloride (0.6 mL) is added to a stirred solution of 1,2,4-triazole (2.1 g, 30 mmol) in 60 mL of acetonitrile, then pyridine (2 mL) is added to dissolve the resulting precipitate. Solution I is added dropwise to Solution II with stirring. After 1 h, amino modified Primer Support (20 g) (Pharmacia) is added in one portion. The suspension is swirled on a rotary shaker for 15 min, then filtered on a sintered glass filter, washed with methanol (200 mL), acetone (500 mL) and ether (200 mL). After being dried for 30 min in vacuo, the support is treated with a mixture of pyridine (60 mL), acetic anhydride (6 mL), and N-methyl imidazole (6 mL). After 15 min, the support is filtered, washed as described above, and dried in vacuo overnight. The product is analyzed for dimethoxytrityl content according to the literature method (Atkinson, T., and Smith, M., in "Oligonucleotide Synthesis, A Practical Approach", M. Gait, Ed., IRL Press, Washington, D. C. pp 35-81 (1984)), and in the specific example was found to have a loading of 32  $\mu\text{mol/g}$ .

Oligonucleotide synthesis. Oligonucleotide synthesis is performed on a Pharmacia OligoPilot DNA synthesizer in 10  $\mu\text{mol}$  scale using either hexanol Primer Support which was prepared accordingly to the

1 procedure described for hexanol CPG (Gamper et al.(1993) Nucleic  
2 Acids Res. 21, 145-150) or hexanoloxylyl Primer Support described  
3 above.

4 For the preparation of oligonucleotides containing  
5 2-thiothymidine and 2-aminoadenosine, two alternative methods can be  
6 used. In the first method, N-phenoxyacetyl protected  
7 5'-O-dimethoxytrityl-2'-deoxynucleoside-2-cyanoethyl-N,N'-diisopropylami  
8 ne-phosphoramidites are used. DNA synthesis cycle is carried out as for  
9 regular phosphoramidites. Time of the deprotection with concentrated  
10 ammonia is reduced to 2 h at 50°C. In the second method, Fmoc  
11 protected phosphoramidites are employed. The synthesis is performed  
12 using the hexanol-oxalyl Primer Support and the standard DNA  
13 synthesis cycle, with the exception of the capping step which is omitted.  
14 Deprotection is carried out by treatment of the solid support with 0.2 M  
15 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU) in DMF for 5 min followed  
16 by 10% ammonia for additional 5 min.



## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Microprobe Corporation  
Bothell, WA 98021
- (ii) TITLE OF INVENTION: SELECTIVE BINDING COMPLEMENTARY  
OLIGONUCLEOTIDES
- (iii) NUMBER OF SEQUENCES: 8
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Klein & Szekeres
  - (B) STREET: 4199 Campus Drive, Suite 700
  - (C) CITY: Irvine
  - (D) STATE: CA
  - (E) COUNTRY: USA
  - (F) ZIP: 92715
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/539,097
  - (B) FILING DATE: 04-OCT-1995
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Szekeres, Gabor L.
  - (B) REGISTRATION NUMBER: 28,675
  - (C) REFERENCE/DOCKET NUMBER: 491-10-PA
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 714-854-5502
  - (B) TELEFAX: 714-854-4897

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 28 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ix) FEATURE:
  - (A) NAME/KEY: misc\_feature
  - (B) LOCATION: 1..28
  - (D) OTHER INFORMATION: /note= "corresponds to "Watson"  
strand of Hybrids I & III"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGACAACGA TCGGAGGACC GAAGGAGC

28

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..28
- (D) OTHER INFORMATION: /note= "corresponds to "Crick"  
strand of Hybrids I & II"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTCCTTCGG TCCTCCGATC GTTGTGAC

28

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: one-of(1, 5, 8, 12, 19, 20, 28)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "pyrrolo-[2,3-d]pyrimidine-2(3H)-one"

(ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: one-of(3, 9, 13, 14, 16, 17, 21, 24, 25, 27)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "hypoxanthine"

(ix) FEATURE:

- (A) NAME/KEY: misc\_feature
- (B) LOCATION: 1..28
- (D) OTHER INFORMATION: /note= "corresponds to "Watson"  
strand of Hybrids II & IV"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NTNANAANNA TNNNANNANN NAANNANN

28

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base  
(B) LOCATION: one-of(1, 9, 10, 17, 21, 24, 28)  
(D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "hypoxanthine"

## (ix) FEATURE:

- (A) NAME/KEY: modified\_base  
(B) LOCATION: one-of(2, 4, 5, 8, 12, 13, 15, 16, 20, 26)  
(D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "pyrrolo-[2,3-d]pyrimidine-2(3H)-one"

## (ix) FEATURE:

- (A) NAME/KEY: misc\_feature  
(B) LOCATION: 1..28  
(D) OTHER INFORMATION: /note= "corresponds to "Crick"  
strand of Hybrids III & IV"

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

NNTNNTTNNN TNNTNNNATN NTTNTNAN

28

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTAAGAGAAT TATGCAGTGC

20

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCACTGCATA ATTCTCTTAC

20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: one-of(2, 10, 11, 13, 18)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "d2sThymine replaces all dThymine"

(ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: one-of(3, 4, 6, 8, 9, 12, 16)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "d2amAdenine replaces all dAdenine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTAAGAGAAT TATGCAGTGC

20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: one-of(3, 8, 10, 11, 19)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "d2amAdenine replaces all dAdenine"

(ix) FEATURE:

- (A) NAME/KEY: modified\_base
- (B) LOCATION: one-of(5, 9, 12, 13, 15, 17, 18)
- (D) OTHER INFORMATION: /mod\_base= OTHER  
/note= "d2sThymine replaces all dThymine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCACTGCATA ATTCTCTTAC

20

**WHAT IS CLAIMED IS:**

1  
2           1.     A pair of oligonucleotides (ODNs), each of said ODNs  
3 comprising nucleotide moieties having naturally occurring aglycon bases  
4 and a combination of modified aglycon bases selected from the group  
5 consisting of the combinations (1) A', T', (2) G', C' and (3) A', T', G',  
6 C', the duplex form of said pair of ODNs having a melting temperature  
7 under physiological conditions of less than approximately 40°C, each of  
8 said pair of ODNs being substantially complementary in the  
9 Watson-Crick sense to one of the two strands of a duplexed target  
10 sequence in nucleic acid,

11           wherein the nucleotide moieties having the modified bases have  
12 the following properties:

13           within complementary oligonucleotides A' does not form a  
14 stable hydrogen bonded base pair with T' and forms a stable hydrogen  
15 bonded base pair with T;

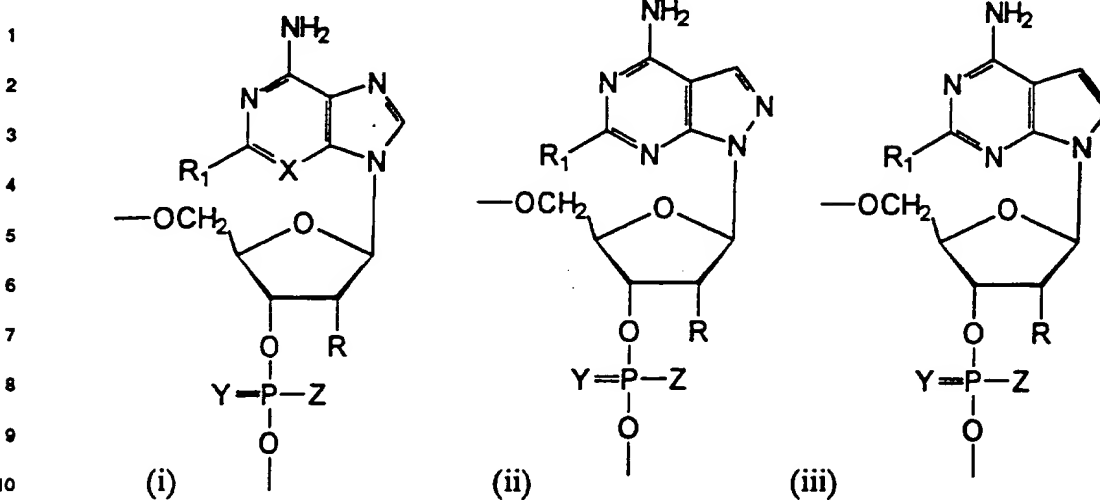
16           within complementary oligonucleotides T' does not form a stable  
17 hydrogen bonded base pair with A' and forms a stable hydrogen bonded  
18 base pair with A;

19           within complementary oligonucleotides G' does not form a stable  
20 hydrogen bonded base pair with C' and forms a stable hydrogen bonded  
21 base pair with C, and

22           within complementary oligonucleotides C' does not form a stable  
23 hydrogen bonded base pair with G' and forms a stable hydrogen bonded  
24 base pair with G, wherein the pair of oligonucleotides are optionally  
25 linked to one another by a covalently bonded tether.

26           2.     The ODNs of Claim 1 wherein the nucleotide moiety A'  
27 has the structure selected from the groups shown by formulas (i), (ii)  
28 and (iii)

59



wherein

X is N or CH;

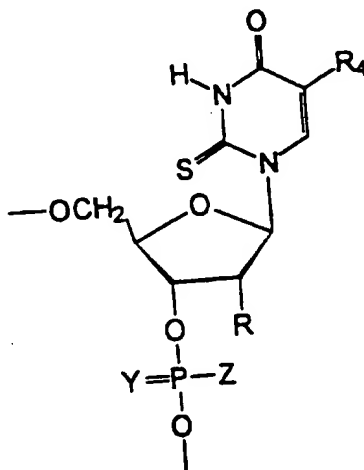
Y is O or S;

Z is OH or CH<sub>3</sub>;

R is H, F, or OR<sub>2</sub>, where R<sub>2</sub> is H, C<sub>1-6</sub> alkyl or allyl, and

R<sub>1</sub> is C<sub>1-4</sub> alkyl, C<sub>1-4</sub> alkoxy, C<sub>1-4</sub> alkylthio, F, or NHR<sub>3</sub>, where R<sub>3</sub> is H, or C<sub>1-4</sub> alkyl, and where the 8 position of the purine, the 3 position of the pyrazolopyrimidine or the 5 position of the pyrrolopyrimidine optionally serve as a point of attachment for a cross-linking function or a reporter group.

3. The ODNs of Claim 1 wherein the nucleotide moiety T' has the formula (iv)



(iv)

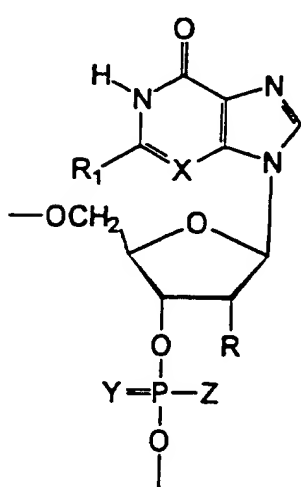
wherein

Y is O or S;

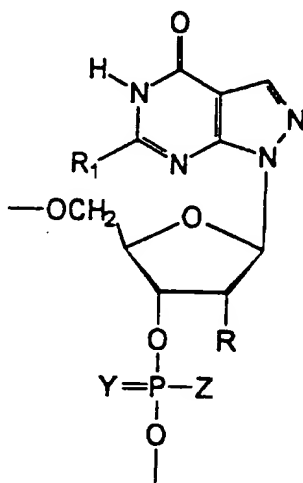
Z is OH or CH<sub>3</sub>;R is H, F, or OR<sub>2</sub>, where R<sub>2</sub> is H, C<sub>1-6</sub> alkyl or allyl, and

R<sub>4</sub> is H, C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkenyl, C<sub>1-6</sub> alkynyl, or optionally the 5-position of the pyrimidine serves as a point of attachment for a cross-linking function or a reporter group.

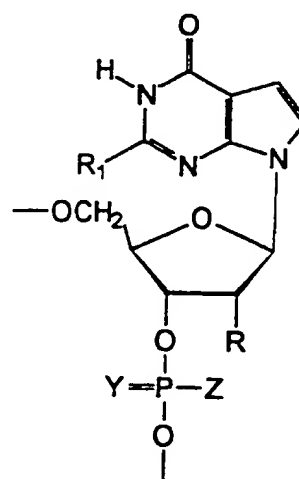
4. The ODNs of Claim 1 wherein the nucleotide moiety G' has the structure selected from the groups shown by formulas (v), (vi) and (vii)



(v)



(vi)



(vii)

wherein

X is N or CH;

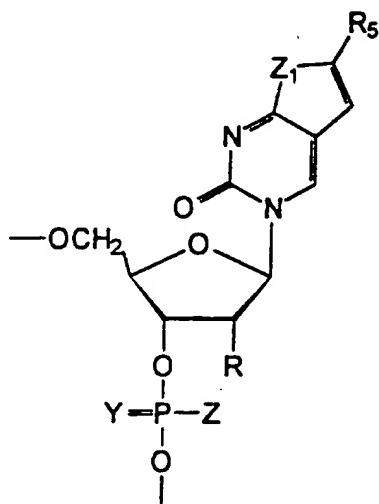
1 Y is O or S;

2 Z is OH or CH<sub>3</sub>;

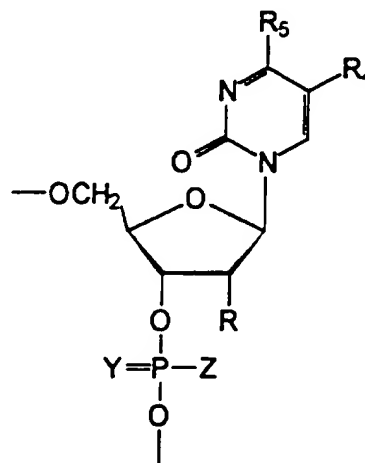
3 R is H, F, or OR<sub>2</sub>, where R<sub>2</sub> is H, C<sub>1-6</sub> alkyl or allyl, and

4 R<sub>1</sub> is H, C<sub>1-4</sub> alkyl, C<sub>1-4</sub> alkoxy, C<sub>1-4</sub> alkylthio, F, or NHR, where  
 5 R<sub>3</sub> is H, or C<sub>1-4</sub> alkyl, and where the 8 position of the purine, the 3  
 6 position of the pyrrazolopyrimidine or the 5 position of the  
 7 pyrrolopyrimidine optionally serve as a point of attachment for a  
 8 cross-linking function or a reporter group.

9 5. The ODNs of Claim 1 wherein the nucleotide C' has the  
 10 structure selected from the groups shown by formulas (viii) and (ix)



(viii)



(ix)

22  
23  
24 wherein

25 Y is O or S;

26 Z is OH or CH<sub>3</sub>;

27 R is H, F, or OR<sub>2</sub>, where R<sub>2</sub> is H, C<sub>1-6</sub> alkyl or allyl,

28 R<sub>4</sub> is H, C<sub>1-6</sub> alkyl, C<sub>1-6</sub> alkenyl, C<sub>1-6</sub> alkynyl, or optionally the  
 29 5-position of the pyrimidine serves as a point of attachment for a  
 30 cross-linking function or a reporter group;



1           Z<sub>1</sub> is O or NH, and

2           R<sub>5</sub> is H, or C<sub>1</sub> - 4 alkyl.

3           6.    The ODNs of Claim 2 wherein the nucleotide moiety A'  
4 has the structure in accordance with formula (i).

5           7.    The ODNs of Claim 6 wherein X is N, Z is OH, and Y is  
6 O.

7           8.    The ODNs of Claim 7 wherein R<sub>1</sub> is NH<sub>2</sub>.

8           9.    The ODNs of Claim 3 wherein Z is OH, and Y is O.

9           10.   The ODNs of Claim 9 wherein R<sub>4</sub> is CH<sub>3</sub>.

10          11.   The ODNs of Claim 4 wherein the nucleotide moiety G'  
11 has the structure in accordance with formula (v).

12          12.   The ODNs of Claim 11 wherein X is N, Z is OH, and Y is  
13 O.

14          13.   The ODNs of Claim 12 wherein R<sub>1</sub> is H.

15          14.   The ODNs of Claim 5 wherein the nucleotide moiety C'  
16 has the structure in accordance with formula (viii).

17          15.   The ODNs of Claim 14 wherein , Z is OH, Z<sub>1</sub> is NH and Y  
18 is O.

19          16.   The ODNs of Claim 15 wherein R<sub>5</sub> is H.

20          17.   The ODNs of Claim 1 having approximately 5 to 99  
21 nucleotide units.

22          18.   The ODNs of Claim 1 wherein each of the nucleotides is a  
23 2'-deoxyribonucleotide.

24          19.   The ODNs of Claim 1 wherein each of the nucleotides is a  
25 ribonucleotide.

26          20.   The ODNs of Claim 1 comprising at least one nucleotide  
27 unit having a 2-O-methylribose moiety.

28          21.   The ODNs of Claim 1 comprising a cross-linking agent  
29 covalently attached to at least one nucleotide unit.

30          22.   The ODNs of Claim 1 comprising a reporter group.

1           **23.**   The ODNs of Claim 1 wherein the combination of  
2   modified aglycon bases is A', T'.

3           **24.**   The ODNs of Claim 1 wherein the combination of  
4   modified aglycon bases is G', C'.

5           **25.**   The ODNs of Claim 1 wherein the combination of  
6   modified aglycon bases is A', T', G', C'.

## INTERNATIONAL SEARCH REPORT

International Application No.

PC/US 96/15934

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07H21/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C07H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STUDIA BIOPHYSICA, vol. 55, no. 1, 1976, pages 21-27, XP002024408 K. H. SCHEIT ET AL: "Stereochemical Basis of Template Function" see page 23 - page 24 see page 22; figure 1 --- -/-	1-3, 6-10, 17-23,25

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*&amp;\* document member of the same patent family

Date of the actual completion of the international search

3 February 1997

Date of mailing of the international search report

10.02.1997

Name and mailing address of the ISA

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Authorized officer

Watchorn, P

## INTERNATIONAL SEARCH REPORT

International Application No

PC./US 96/15934

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHEMICAL ABSTRACTS, vol. 108, no. 21, 23 May 1988 Columbus, Ohio, US; abstract no. 187183, H. INOUE ET AL: "Synthesis of Dodecadeoxyribonucleotides Containing a Pyrrolo[2,3-d]pyrimidine Nucleoside and their Base-Paring Ability" page 752; column 1; XP002024262 see abstract & NIPPON KAGAKU KAISHI, no. 7, 1987, pages 1214-1220, ---	1,4,5, 11-22, 24,25
A	DATABASE WPI Section Ch, Week 8750 Derwent Publications Ltd., London, GB; Class B02, AN 87-352165 XP002024264 & JP 62 255 499 A (TEIJIN KK) , 7 November 1987 see abstract & PATENT ABSTRACTS OF JAPAN vol. 012, no. 139 (C-491), 27 April 1988 & JP 62 255499 A (TEIJIN LTD), 7 November 1987, see abstract ---	1,4,5, 11-22, 24,25
A	NUCLEIC ACIDS RESEARCH, vol. 22, no. 2, 25 January 1994, OXFORD GB, pages 131-136, XP002024256 S. CASE-GREEN ET AL: "Studies on the base pairing properties of deoxyinosine by solid phase hybridisation to oligonucleotides" see the whole document ---	1,4,5, 11-22, 24,25
A	NUCLEIC ACIDS RESEARCH, vol. 13, no. 24, 20 December 1985, OXFORD GB, pages 8927-8938, XP002024257 F. H. MARTIN ET AL: "Base pairing involving deoxyinosine: implications for probe design" see the whole document ---	1,4,5, 11-22, 24,25
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## INTERNATIONAL SEARCH REPORT

International Application No

PC/US 96/15934

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>NUCLEIC ACIDS RESEARCH, vol. 16, no. 1, 11 January 1988, OXFORD GB, pages 305-317, XP002024258 A. CHOLLET ET AL: "DNA containing the base analogue 2-aminoadenine: preparation, use as hybridization probes and cleavage by restriction endonucleases" see the whole document ---</p>	1-3, 6-10, 17-23,25
A	<p>WO 95 14707 A (ISIS PHARMACEUTICALS INC) 1 June 1995  see page 11, paragraph 2 ---</p>	1-3, 6-10, 17-23,25
A	<p>NUCLEIC ACIDS RESEARCH, vol. 22, no. 8, 25 April 1994, OXFORD GB, pages 1429-1436, XP002024259 R. KUIMLEIS ET AL: "Synthesis of oligodeoxynucleotides containing 2-thiopyrimidine residues - a new protection scheme" see page 1430, compounds 1a, 1b; page 1432, table 1 and page 1435, column 1, paragraph 3 ---</p>	1-3, 6-10, 17-23,25
A	<p>CHEMICAL ABSTRACTS, vol. 116, no. 13, 30 March 1992 Columbus, Ohio, US; abstract no. 129471, T. ISHIKAWA ET AL: "Synthesis and Properties of Oligothymidylate Containing Sulfur-Modified Thymidine : Effect of Thiation of Pyrimidine Ring on Thermostability and Conformation of the Duplex" page 949; column 2; XP002024263 see abstract &amp; BIOORG. MED. CHEM. LETT., vol. 1, no. 10, 1991, pages 523-526, --- -/--</p>	1-3, 6-10, 17-23,25

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/15934

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BIOCHEMISTRY, vol. 29, no. 42, 23 October 1990, EASTON, PA US, pages 9891-9901, XP002024260 P. C. NEWMAN ET AL: "Incorporation of a Complete Set of Deoxyadenosine and Thymidine Analogues Suitable for the Study of Protein Nucleic Acid Interactions into Oligonucleotides. Application to the EcoRV Restriction Endonuclease and Modification Methylase" see page 9895; figure 2 see page 9896; tables I,II ---</p>	<p>1-3, 6-10, 17-23,25</p>
A	<p>JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 113, no. 13, 19 June 1991, DC US, pages 5109-5111, XP002024261 P. L. RICHARDSON ET AL: "Tethered Oligonucleotides. A Strategy for the Recognition of Structured RNA" see the whole document ---</p>	<p>1-25</p>
A	<p>WO 95 05391 A (CHROMAGEN INC) 23 February 1995 see figure 14 ---</p>	<p>1-34</p>
P,X	<p>NUCLEIC ACIDS RESEARCH, vol. 24, no. 13, 1 July 1996, pages 2470-2475, XP000621694 WOO J ET AL: "G/C-MODIFIED OLIGODEOXYNUCLEOTIDES WITH SELECTIVE COMPLEMENTARITY: SYNTHESIS AND HYBRIDIZATION PROPERTIES" see the whole document -----</p>	<p>1,4,5, 11-18, 21,24</p>

# INTERNATIONAL SEARCH REPORT

I national application No.

PCT/US 96/15934

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
Claims searched incompletely: 1-22  
Please see attached sheet ./,
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC./US 96/15934

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9514707	01-06-95	US-A-	5459255	17-10-95
		EP-A-	0731807	18-09-96
		US-A-	5587469	24-12-96
-----				
WO-A-9505391	23-02-95	CA-A-	2145750	23-02-95
		EP-A-	0669928	06-09-95
-----				